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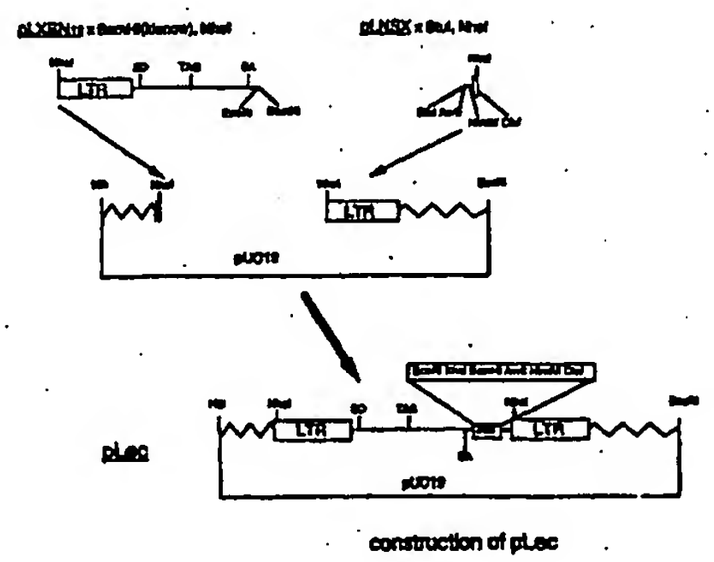
(54) Title: IMPROVED RETROVIRAL VECTORS, ESPECIALLY SUITABLE FOR GENE THERAPY

(57) Abstract

The present invention relates to the field of molecular biology, especially recombinant DNA technology, especially concerning retroviral vectors. Retroviral vectors are very suitable vehicles for transferring genetic material of interest into certain cells in so-called gene therapy strategies. However, the retroviral vectors described so far are not ideal. They may give rise to recombination events resulting in helper (pathogenic) virus, they may express viral proteinaceous materials leading to immune responses, etc. These and other drawbacks are overcome by the vectors, cells, kits and methods of the present invention by providing a vector derived from a retrovirus, comprising a sequence responsible for transcriptional control, including an enhancer, which vector further comprises a site for insertion of at least one gene of interest, a packaging signal, said vector having no superfluous retroviral sequences and no open reading frame encoding at least parts of viral proteins, characterized in that the enhancer is an enhancer that is active in undifferentiated cells.



construction of pLec

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Title: Improved retroviral vectors, especially suitable for gene therapy.

The invention relates to improved retroviral vectors which are especially useful for methods of gene therapy. The invention further relates to such vectors in combination with suitable packaging cell lines, as well as virus-like particles which can be produced using said combination and methods of providing cells with genetic information of interest using said virus-like particles.

Retrovirus-based vectors are highly favoured tools to achieve stable integrated gene transfer of foreign genes in mammalian cells. Especially in the area of gene therapy their use has attracted considerable attention. Retrovirus-based vectors have been used in both *ex vivo* and *in vivo* gene transfer procedures and were shown to be capable of yielding long-term expression of foreign genes in culture and *in vivo* in animal studies as well as in man.

Retroviral vector systems for (safe) gene therapy purposes comprise two building blocks:
the recombinant retroviral vector that carries the genetic information which is to be transduced plus all of the elements required *in cis* for the packaging and integration of the viral genome, and; retroviral packaging cells that provide the viral proteins encoded by the genes *gag*, *pol* and *env*. These polypeptides are required *in trans* for the production of viable virus particles but by themselves, the packaging cells are incapable of releasing infectious virus. Packaging cells transduced with the recombinant vector will therefore generate recombinant retroviruses carrying the genetic information contained in the aforementioned vector. These viruses can subsequently be used to transduce cells in which the recombinant material will be integrated following the natural retrovirus life cycle. The current invention in one aspect relates to recombinant vectors with improved characteristics as compared to the previously described vectors.

A favoured design of the recombinant virus with properties that permit long term expression in animals and which is based on a non-pathogenic retrovirus, has previously been described (Valerio *et al.*, 1989; International patent application WO9307281). In these constructs the gene of interest is under the transcriptional control of a viral long terminal repeat (LTR). In the favoured LTR the retroviral enhancer is replaced by an enhancer active in undifferentiated cells such as embryo carcinoma (EC) cells. An example is the enhancer of a polyoma virus mutant (PyF101) that was selected to grow on undifferentiated EC cells. This results in mutations in its enhancer that permit activity of heterologous promoters in primitive cells. An LTR based on Moloney murine leukemia virus (Mo-MLV) in which the enhancer is replaced for by the PyF101 enhancer is called ΔMo+PyF101. Since replication competent viruses harbouring the ΔMo+PyF101-LTR can cause viremia in newborn mice <u>without</u> causing disease, the properties of ΔMo+PyF101-carrying vectors are superior to the commonly used vectors based on viral backbones of disease-inducing viruses such as Moloney murine leukemia virus, myeloproliferative sarcoma virus or Harvey murine sarcoma virus (Davis *et al.*, 1985).

Our work with ΔMo+PyF101-based retroviral vectors has led to the conclusion that they are capable of introducing genes into primary haemopoietic stem cells of mice, non-human primates and man. Following transplantation of such modified stem cells sustained expression of the transduced gene was observed in all haemopoietic lineages analyzed in the absence of any gene transfer-related toxicity (Einerhand *et al.*, 1991; Van Beusechem *et al.*, 1990, 1992, 1993, 1994 and 1995). Based on these results the world first clinical gene therapy study in man aimed to correct the haemopoietic stem cells of three patients with an inherited deficiency of adenosine deaminase was performed using the aforementioned retroviral vector carrying a correct version of the human adenosine deaminase gene (Hoogerbrugge *et al.*, 1995).

Despite the many favourable characteristics of the previously described vector, several limitations of the

original design will still limit its general usefulness as a gene therapy product.

Identification of these shortcomings as well as remedies for these shortcomings are one aspect of the present invention. These shortcomings of the recombinant vectors are:

1) The presence of superfluous retroviral sequences which do not contribute to the efficient packaging of the recombinant virus, which limit the space available for genes to be transduced and which increase the risk of recombination events during the generation of producer cells possibly leading to the production of helper virus.

2) The presence of a *gag* open reading frame which may result in the translation of truncated viral proteins that would cause unwanted immune responses and will probably increase the potential of helper-virus formation in packaging cells carrying homologous *gag* sequences.

3) The presence of superfluous non-coding sequences of the gene(s) of interest, limiting space and possibly negatively affecting gene expression and/ or messenger stability.

4) Expression *in vitro* and *in vivo* is still influenced by the integration site (e.g. Einerhand *et al.*, 1993) and therefore variable. The inclusion of elements that could control site independent expression such as specific boundary elements (Chung *et al.*, 1993) and Locus Control Regions (Grosveld *et al.*, 1987; Greaves *et al.*, 1989/WO 9101329) have been suggested, but were shown to be the cause of rearrangements (e.g. Novak *et al.*, 1990).

5) Sequences contained within the viral construct can function as unwanted cryptic splice sites, resulting in packaging and expression of aberrant RNA molecules. (e.g. McIvor *et al.*, 1987; Sorrentino *et al.*, 1993).

6) Given that in the preferred configuration of the vector the gene of interest is under transcriptional control of the viral LTR, the expression of more than one gene can only be achieved through bicistronic messengers or in the form of fusion proteins. Currently used intercistronic sequences are derived from viruses (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988), they are in general large and contain extensive

secondary structures which render them less favourable than the synthetic intercistrons according to the present invention.

The problems as mentioned under nos. 1 and 2 have been solved in the prior art, in vectors designated as the LN series (Miller and Rosman, 1989), but despite the large body of work on retroviral vectors that has been published since the availability of the LN-based vectors, to our knowledge, they were never combined with constructs in combination with ΔMo+PyF101 or other enhancer-replaced LTRs.

The current invention discloses a basic vector and derivatives thereof that will result in improvements as related to the shortcomings stated above.

Thus, the current invention provides, in one aspect, a vector derived from a retrovirus, comprising a sequence responsible for transcriptional control, including an enhancer, which vector further comprises a site for insertion of at least one gene of interest, a packaging signal, said vector having no superfluous retroviral sequences and no open reading frame encoding at least parts of viral proteins, characterized in that the enhancer is an enhancer that is active in undifferentiated cells.

The vectors according to the invention have all the benefits of the so-called LN vectors as earlier mentioned, but they have the additional advantage that they can be used to functionally transfer material into undifferentiated cells and retaining said function throughout the differentiation and other processes. Preferably the modified LTR comprising the invented enhancer will not be able to give rise to significantly pathogenic viruses. The ΔMo+PyF101 enhancer is a good example of such an enhancer and is a preferred embodiment of the present invention. Based on the disclosure of the present invention however, the skilled worker will be able to design other similarly suitable enhancers. In designing vectors according to the invention, the skilled worker will be able to modify the concept of the invention in order to suit his needs. Therefore it will be clear that the definitions of the present application should be interpreted in a broad sense. For instance a gene will read upon any DNA-like material to be transduced into a cell.

Thus non-coding DNA or cDNA are within that definition. Similar defintions shoud be given a similar scope. The skilled worker will now also be able to work around the problems or shortcomings as identified above, because once identified according to the invention, in most cases the solution presents itself. For instance, now that a problem has been identified in that a viral sequence may contain a cryptic splice site, the skilled worker can examine the viral sequence for such a site and delete or mutate it. The same is of course true for untranslated parts of the gene of interest. The skilled worker may now identify such untranslated superfluous sequences and remove them from the gene of interest.

The advantages to be gained by providing a short stretch of intercistronic linker-basepairs, which has a number of bases dividable by three is clear to the skilled worker.

The other characteristics of the vectors according to the invention as defined in the claims and the specification by themselves or in combination lead to clear advantages over the prior art. Insofar as they have not been clearly defined herein, they are clear to the skilled workers in the field.

Of course the ultimate goal of the vectors according to the invention lies in providing a safe and viable system of providing certain subsets of cells with additional genetic material, especially in the context of gene therapy.

For efficient transfer of the vectors according to the invention it is usually necessary that they be presented in a virus-like infectious particle. For providing such a particle it is of course necessary that a packaging signal is present on the vectors. Such a packaging signal may be any functional one, i.e. one that works with the packaging material. The packaging material will usually be provided by a cell into which the vector is transferred. Said packaging material will of course have to be functional in packaging the vectors according to the invention. The most logical and most preferred combination is that of the retroviral packaging signal together with a cell that constitutively produces the retroviral proteins necessary for packaging, a so-called packaging cell line. The many possible combinations of the two (kit of parts) are of course part of the present invention.

Both the positive mode and the negative mode of gene therapy can be realized using the vectors according to the invention. For the present application the positive mode of gene therapy is intended to read upon any deficiency in a group of cells that can be treated by providing at least a number of said cells with a gene capable of removing said deficiency, such as for instance providing hematopoietic cells with a gene encoding factor VIII for correcting haemophilia. The negative mode of gene therapy for this application includes the functional removal of any subset of cells within an individual by introducing genetic material into at least a number of cells from the subset. Examples are suicide genes for tumour cells.

Description of specific embodiments

In one aspect the invention relates to several retroviral vectors that share a number of basic characteristics and that can be used to efficiently generate infectious recombinant virus particles when transfected into packaging cells. Hereunder a description of the basic embodiments present in the vectors according to this invention is given, as well as the specific characteristics of some of the preferred retroviral vectors that are included in this invention together with examples of their applications.

The described retroviral vectors contain a 5' LTR, preferably from Moloney murine sarcoma virus (MoMSV base 1 to 541; numbering according to Van Beveren et al., 1985), including part of the packaging signal. The remainder of the packaging signal is preferably derived from Moloney murine leukemia virus (MoMLV base 566 to 1038) with the start codon of the *gag* coding domain preferably mutated to a stop codon (Miller and Rosman, 1989). The LTR and the extended packaging signal with the point mutations together ensure efficient packaging of the recombinant virus whithout any production of virus-derived proteins in the target cells. Furthermore, the reduced sequence homology between the MoMSV LTR and the 5' part of the packaging constructs (MoMLV LTR) generally used in packaging cells like PA317 and PG13 will reduces the chance of recombination between the constructs and thus reduces the chance of helper virus formation. A splice donor site located downstream of the 5' LTR can in combination

with the splice acceptor site just upstream of the insertion site of the gene of interest lead to enhanced translation of the inserted gene in case splicing occurs. To further reduce chances of recombination of retroviral vectors with packaging constructs, the vectors contain no overlap with the 3' end of the *env* constructs used in the above mentioned packaging cell lines. Such a construct is again a preferred embodiment.

The resulting vector (shown in Figure 1) having all preferred features, contains a 5' and 3' LTR, a packaging signal extending into the *gag* coding region and a poly cloning site for the insertion of (a) gene(s) of interest and thus meets the basic requirements a retroviral vector according to the invention should meet.

In another preferred embodiment, the above basic retroviral vector is modified by deleting (parts of) the *gag* coding sequences without losing packaging function. Such a vector further reduces the probability of recombination events in the packaging cells that may lead to replication competent retroviruses. Thus this improves the general safety features of the retroviral vectors.

This invention also discloses further modifications to this basic retroviral vector to allow more efficient transcription and translation. First, a consensus Kozak sequence is introduced around the ATG of the gene of interest to improve translation of that gene. Second, the viral enhancer in the 3' LTR is replaced by a mutant form of the Polyoma virus enhancer that is specifically selected for activity in F9 embryonal carcinoma cells (Linney *et al.*, 1984) and that is known to be less sensitive for promoter inactivation in haemopoietic stem cells and early haemopoietic progenitor cells compared to the wild type MoMLV enhancer (Valerio *et al.*, 1989; Van Beusechem *et al.*, 1990). Another advantage arising from the replacement of the wild type MoMLV enhancer for the polyoma enhancer is that the resulting ΔMo+PyF101 LTR renders murine leukemia viruses into non-pathogenic viruses (Davis *et al.*, 1985). Following one round of replication this alteration contained within the U3 region of the 3' LTR is also tranferred to the 5' LTR and is thus present in both LTR's of the proviral sequence.

As a consequence, after infection of an amphotropic packaging cell line, the ΔMo+PyF101 LTRs contain less sequence homology with the packaging constructs as compared to LTRs with wtMoLV enhancers.

Additional modifications to improve expression in target cells include:

- incorporation of a Locus Control Region (LCR) e.g. the CD2 LCR for high and controled expression in T-cells (see example 5).
- Incorporation of a selectable marker preferably as a second gene in a dicistronic transcription unit. Selection genes include the neomycine^r gene, the hygromycine^r gene, a gene encoding a fluorescent protein or a gene coding for a biologically inactive transmembrane molecule that all allow for efficient selection *in vitro*. Alternatively, selection markers may be included that also allow for selection of transgene expression *in vivo* like for example, without limitation, the human genes for Multi Drug Resistance (MDR-1 see examples 2 and 3), a UDP-Glucuronosyl Transferase, Thymidylate Synthetase, canalicular Multispecific Organic Anion Transporter, γ-Glutamyl Cysteine Synthetase, as well as biologically active mutants of these genes.
- additional (regulatory) sequences that can influence the expression of the integrated provirus in the target cells e.g. boundary elements and/or (tissue-specific) promoters or enhancers.

Preferred or additional properties of the retroviral vectors described in this invention include:

- a gene of interest with no or a minimum of 5' and 3' non-translated sequences necessary for maximum RNA stability and translation.
- a dicistronic transcription unit whereby the two coding regions are separated by a short non-coding linker allowing efficient reinitiation of the ribosomal complex on the start codon of the second gene. This non-coding linker can have a variable length but is devoid of any ATG sequences or sequences that form strong secondairy structures in the RNA. For maximum efficiency of translation, the length of a favourable intercistron is a multiplicity of 3, the stop codon of the first

gene thus placed in frame with the start codon of the second gene.

Construction of the retroviral vector pLec

The retroviral vector pLXSN (Miller and Rosman, 1989) was digested with NheI and the insert containing the viral sequences was ligated into the vector backbone pUC19 obtained from pSFG-tpa (R. Mulligan and I. Riviere, Whitehead Institute for Biomedical Research, Cambridge, MA) after digestion with NheI. The resulting construct, named pLXSN19, was digested with BamHI and the ends were filled in using the Klenow enzyme. After removal of the enzymes, the DNA was digested with NheI after which the 1452 bp NheI/BamHI fragment containing the 5' LTR and the extended packaging signal was isolated. A 98 bp fragment containing the 3' LTR was isolated from the vector pLNSX (Miller and Rosman, 1989) following digestion with NheI and StuI. Ligation of these fragments into the NheI fragment from pSFG-tpa containing the pUC19 backbone resulted in the viral construct pLec (Figure 1).

Modification of the viral enhancer in the 3' LTR of pLec

pLec was digested with NheI and the fragment containing the viral sequences was ligated into the NheI site of pSK/ZipΔMo+PyF101 resulting in pLecΔMo (Figure 2). This vector carries the modifications in its 3' LTR which will result in conversion of these alterations into the 5' LTR after one round of replication (Valerio *et al.*, 1989). pSK/ZipΔMo-PyF101 has been generated by subcloning of the ClaI-EcoRI fragment from pZipΔMo+PyF101(N$^-$) into the pBluescript vector (M. Einerhand, TNO, Radiobiological Institute). pZipΔMo+PyF101(N$^-$) is a low copy vector (pBR 322 based) containing a 3' LTR that has been made by combining the ClaI/KpnI fragment from pMLV-C/R/BΔMo+PyF101 (Linney *et al.*, 1984) with a KpnI/ EcoRI fragment from pZipSV(X)1 (Cepko *et al.*, 1984). The first contains part of the R region and a U3 region in which the enhancer sequences have been replaced by a mutant form (F101) of the Polyoma virus enhancer, the second contains the remaining R and U5 sequences of the 3' LTR. After destroying the NheI site in the tetracycline^r gene in the vector sequences, the unique

NheI site can be used to swap recombinant vectors with wild type enhancers into one that contains the mutant form (Valerio et al., 1989).

Example 1: Monocistronic and bicistronic retroviral vectors for suicide gene therapy

A promising method for the treatment of solid tumours is the introduction of suicide genes into the tumour cells *in vivo*. Suicide genes, like the Herpes simplex virus type 1 thymidine kinase (HSV-tk) gene or the cytosine deaminase gene, encode proteins that are capable of transferring a non-toxic prodrug into a toxic drug. For example the prodrug ganciclovir is not toxic for eukaryotic cells but after (mono)phosphorylation by the HSV-tk gene it will be converted into a nucleotide analog by cellular enzymes. Incorporation of this analog into the DNA of replicating cells results in chain termination and cell death. The attractivity of this system as an anti tumour therapy has become apparent from the notice that transduced cells that are dying due to the prodrug treatment can trigger death of untransduced cells in their close vicinity: 'the bystander effect' (Moolten, 1986). Several groups have used tumour models in rats to show that after transfer of a suicide gene into tumour cells *in vivo*, only a minority of the tumour cells need to express the suicide gene in order to establish an effective anti-tumour respons (reviewed in Moolten, 1994). Efficient transduction of tumour cells can be achieved *in vivo* by direct injection of retrovirus producer cells into the tumour (Culver *et al.*, 1992).

As an example for the application of the retroviral vectors outlined in this invention we describe the vector pIGTk and its use in suicide gene therapy for malignant brain tumours in a rat model.

Construction of pIGTk

The retroviral vector pLec was digested with XhoI, blunted with Klenow and subsequently digested with BamHI. After dephosphorylation using calf intestine phosphatase (CIAP), this fragment was ligated to a fragment containing the coding region of the Herpes simplex virus type 1 thymidine kinase gene (HSV-1

tk) obtained from pAdTK (Bram Bout, IntroGene; European Patent Application 94202322.7) by digestion with HindIII, blunting with Klenow and digestion with BamHI. The resulting viral vector was named pLTk.

pLTk was linearised with BsiWI, partially digested with EcoRI and dephosphorylated with CIAP. The 5' part of the tk gene was then reintroduced as a EcoRI/BsiWI pcr fragment obtained after amplification of the 5' part of the tk gene in the vector TNFUS69 (Schwartz *et al.*, 1991) using the primers TKkozUp: 5'-CGGAATTCGCCGCCACCATGGCTTCGTACCCCGGCCATCAG-3' and TkDo-1: 5'-CGGCTCGGGTACGTAGACGATATCG-3' followed by digestion with EcoRI and BsiWI. The resulting retroviral construct was named pLTKkoz (Figure 3). Using this strategie for cloning, a retroviral vector was created that only contains the coding sequences of the HSV-1 tk gene with an optimized Kozak sequence around the start codon. The NcoI site 5' and the BamHI, AvrII, HindIII and ClaI sites 3' of the inserted gene are useful cloning sites to swap inserts in this vector (Figure 4a).

The NheI fragment from pLTKkoz was introduced into the unique NheI site from pSK/ZipΔMo+(PyF101) to generate construct pIGTk (Figure 4b).

The retroviral vector pIGTk was cotransfected into the amphotropic packaging cell line PA317 (ATTC No. CRL 9078) together with an expression construct containing the neomycine^r gene (ΔMo+PyF101LTR-Neo, M. Einerhand unpublished) and G418 resistant clones were isolated. One of these produced around $1x10^5$ infectious virus particles/ml that were capable of transferring the HSV-1 tk gene to thymidine kinase deficient Rat-2 cells. This virus producer (termed IG-RV-TK) has been succesfully used in a preclinical study aimed at curing experimental brain tumours in rats (Vincent *et al.*, 1996). In this study 344 Fischer rats were inoculated with $4x10^4$ 9L rat gliosarcoma brain tumour cells (Weizsaeker *et al.*, 1981) in the left forebrain using a stereotaxic apparatus. After 3 days the growing tumours were inoculated once with $5x10^6$ IG-RV-TK producer cells, $5x10^6$ control cells (PA317 non-producer cells, IL-2 retrovirus producer cells or LacZ retrovirus producer cells), supernatant from IG-RV-TK cells or PBS. Treatment with ganciclovir 15 mg/kg twice daily intraperitoneally for ten days

was initiated 8 days after inoculation of the 9L tumour cells. Figure 5 clearly shows the prolonged survival of rats treated with IG-RV-TK producer cells in combination with ganciclovir (Vincent *et al.*, 1996).

The tk/ganciclovir system can also be of great value in the treatment of leukemia. Currently, patients treated for leukemia often receive an allogeneic bone marrow transplantation (BMT), using a BM graft from an (un)related, but closely MHC-matched donor. The presence of T-cells in such a graft has turned out to be a major factor determining the succes of the treatment. In addition, it has been demonstrated that in patients receiving an allogeneic BMT the likelihood of a leukemia relapse is reduced due to a so called graft-versus-leukemia (GVL) reaction (Antin, 1993). However, these patients often suffer from a severe life-threatening graft-versus-host disease (GVHD). Unfortunately, it seems not possible to separate the GVL and GVHD reactions in human patients. Thus, although the patients clearly benefit from the presence of allogeneic T-cells in the graft, this treatment is seriously hampered by the occurrence of GVHD. A solution to this problem could be to isolate (allogeneic) peripheral blood lymphocytes from the donor prior to the BMT, transduce them *in vitro* with a suicide gene and use these cells together with the T-cell depleted BM graft (Tiberghien *et al.*, 1994). In case GVHD develops treatment of the patients with ganciclovir will result in selective killing of the activated (transduced) T-cells prospectively leading to abrogation of GVHD. Using this method patients may still benefit from a GVL reaction resulting in a decreased rate of leukemia relapse.

A prerequisite for the success of this approach is that virtually all T-cells that are infused into the patients stably express the suicide gene to be able to eradicate them *in vivo* with ganciclovir. The efficiency by which human peripheral blood lymphocytes can be transduced *in vitro* with retroviral vectors is unlikely to ever become 100%. As a consequence it is necessary to incorporate a selection marker in the retroviral vector. In former experiments with retroviral constructs harboring an internal promoter driving the expression of a second gene (often the selection gene), it was noticed that interference between the 2 promoters in the retrovirus often

resulted in (unwanted) shut-off of one of the promoters (Emerman and Temin, 1984, 1986). Therefore, in the constructs described in this invention both genes are located in a dicistron driven by one (viral) promoter. Dicistronic mRNAs allow for efficient translation of the second gene if the genes are separated by either a short intercistronic linker depending on ribosome scanning, or by specialised sequences triggering internal binding of ribosomes. According to the ribosome scanning model (Kozak, 1987a, 1989) the small ribosomal subunit binds to the 5' end of the capped mRNA and scans for the presence of ATG sequences. Initiation of translation occurs at the first ATG in a favourable context (Kozak, 1987b) and the translation complex dissociates when a stop codon is encountered. Translation of a second coding region is possible presumably because the small subunit (or another factor of the elongation complex) continues to scan along the mRNA and, when an ATG is recognised, is able to reinitiate translation. Experiments using different intercistronic linker sequences have shown that the sequence itself is of more importance than the length (Kozak, 1987a; Levine *et al.*, 1991). The use of internal ribosomal entry sites (IRES) from picornaviruses to express two genes in a dicistron following retroviral infection has been described by W. Anderson in patent application WO 9303143. These viral sequences are however relatively large and have strong secondary structures which could affect the packaging capacity and stability of the construct and its RNA product.

As an example of a favourable dicistronic mRNA we describe here the construct pLTk+NeoΔMo in which the tk gene and the neo^r gene are separated by a 36 nucleotide linker. The tk or the neo^r gene can both be replaced by other genes. In general, a favourable intercistronic linker: (1) Has the start codon of the second gene inserted in frame with the stop codon of the first gene; the length thus being a multiplicity of 3, (2) should not contain any ATG sequences, (3) can vary in length between 9 and 200 bp and (4) should not contain sequences that form strong secondary structures in the RNA. The two genes with their intercistrons can subsequently be introduced into the retroviral construct.

Construction of pLTk+NeoΔMo

The HSV-tk gene was excised from pLTKkoz using EcoRI and BamHI and subcloned into pUC119. An EcoRI/HincII fragment containing the promoter of the human phosphoglycerate kinase gene (Singer-Sam *et al.*, 1984; Michelson *et al.*, 1983) was provided with an EcoRI linker and introduced into the EcoRI site generating pPGK-Tk. To optimise translation of the neo^r gene and introduce an NcoI site at the start of the neo^r gene, the sequence around the ATG codon was changed into the consensus Kozak sequence (Kozak, 1987b) by a pcr reaction on pMC1neopA (Thomas and Capecchi, 1987) using the primers 5'-CCCTGCAGCGCCACCATGGGATCGGCCATTGAACAAGATGG-3' (forward) and 5'-GCCAGTCCCTTCCCGCTTC-3' (reverse). The 280 bp pcr fragment was digested with PstI and subcloned into the pBluescript KS$^-$ vector (Stratagene). A PstI/AsuII fragment from pLNCX (Miller and Rosman, 1989) containing the 3' part of the neo^r gene was then introduced into this vector after digestion with ClaI followed by dephosphorylation and partial PstI digestion. The modified neo^r gene was isolated as a BamHI/SalI fragment and cloned into the corresponding sites in pPGK-TK, resulting in pPTk+Neo/IF. Lastly, a blunted EcoRI/BamHI fragment from pAMG-1 (Valerio *et al.*, 1985) containing an poly Adenglation signal from hepatitisB virus (HBV) was introduced into the blunted HindIII site, generating pPTk+NeopA/IF. In this construct the stop codon of the tk gene is positioned in frame with the start codon of the neo^r gene. Using a different forward primer in the pcr reaction, lacking the C just 3' from the PstI site, a second Tk-Neo dicistron has been generated in which the two coding regions are separated by a 35 nucleotide linker in stead of 36 nucleotides (pPTK-NeopA/OF; Figure 6).

The Tk+Neo dicistron from pPTk+NeopA/IF was excised by EcoRI and SalI digestion and ligated into the pLec vector after digestion with EcoRI (partial) and XhoI. pLTk+NeoΔMo was completed after subcloning of the NheI fragment into the corresponding site in pZipΔMo+PyF101(N$^-$).

Efficient translation of the second gene in a bicistron

Two monocistronic (control) constructs were generated carrying either a single TK coding region (pPTkpA) or the neo^r coding region (pPNeopA; Figure 6). The first originated after digestion of pPTk+NeopA/IF with BamHI and SalI, blunting with Klenow enzyme and religation, the second after partial digestion with NcoI and religation of the vector fragment lacking the tk gene. To test the efficiency of translation of both coding regions in the bicistronic constructs, plasmid DNA (16 µg) was cotransfected with 4 µg pCMVLuc (L. Fortunati and M. Scarpa, unpublished) using the $CaPO_4$ coprecipitation method described by Chen and Okayama (1987). Addition of equal amounts of the luciferase expression construct to each DNA/$CaPO_4$ mixture enables correction for differences in transfection efficiency. Two days after transfection cells were trypsinised and half was used to measure luciferase activity. The other half of the cells was partly plated in different dilutions (1/100 and 1/500) in 6 cm dishes and selected for either tk (HAT-supplemented medium) or neo^r (medium plus 1mg/ml G418 sulphate) activity and the remaining cells were pooled and selected for neo^r activity (except for the transfection with pPTkpA wich was selected in HAT supplemented medium). Table 1 shows the relative number of colonies obtained after selection in HAT or G418 containing medium and correction for the luciferase activity. From these results it is clear that the tk activity in the bicistronic constructs is comparable whereas the translation of the second gene (neo^r) is less efficient when the first and the second gene are not in frame with each other.

	# of colonies corrected for luciferase activity	
	HAT selection	G418 selection
pPTk+NeopA/IF	12 5	16 12
pPTk-NeopA/OF	11 8	2 3
pPNeopA	- -	61 96
pPTkpA	5 8	- -

Table 1: Relative number of colonies obtained after transfection of the different constructs into Rat-2 cells grown in indicated selective medium. Each transfection has been performed in duplo. See text for details.

The G418 selected pools containing the constructs pPTk+NeopA/IF, pPTk-NeopA/OF or pPNeopA and the HAT selected pool pPTkpA can be used to test the sensitivity of the cells to the prodrug ganciclovir. To test this, cells were plated at low density ($1x10^4$ cells) in 75 cm^2 flasks in the presence of 5 µM Ganciclovir (GCV). Growing colonies were scored and represent tk^- cells present in the pools of transfected cells. Cells were plated at low density to avoid negative influences of the bystander effect i.e. the death of untransduced cells due to the transmittance of the toxic activity from transduced cells. Plating efficiency was determined by mixing increasing numbers of tk^- cells (100- 5000) with or without $1x10^4$ tk^+ cells and culture in medium containing 5 µM GCV. From these experiments it became clear that the pool of cells transfected with pPTk-NeoPA/IF contained -12% tk^- cells whereas the pool of cells

transfected with pPTk-NeopA/OF contained -45% tk^- cells. The appearance of tk^- cells can be partly explained by the fact that circular plasmid DNA was used for the transfection resulting in integration events that inactivate the tk gene. The difference between the two bicistronic constructs however, can only be explained by differences in translation of the neo^r gene due to the different intercistronic linker. The outcome of the above described experiments lead to the conclusion that the situation in which the two coding regions in a bicistron are placed in frame to each other is favourable compared to an out -of- frame situation.

To be able to directly compare the performance of the bicistronic constructs with an intercistronic linker sequence with bicistronic constructs containing an IRES sequence, two additional bicistronic expression constructs were made. The first, pPTkpolioNeo, contains an IRES sequence derived from poliovirus and the second, pPTkemcvNeo containes an IRES sequence from EMCV (encephalomyocarditis virus). pPTkpolioNeo was generated by ligation of a 750 bp Klenow treated HindIII-EcoRV fragment from pP2-5' (Pelletier *et al.*, 1988) into pPTk+NeopA/IF digested with XbaI followed by Klenow treatment. The resulting construct was then digested with BamHI and partially digested with NcoI, Klenow treated and religated to remove most of the linker sequences present in pPTk+NeopA. pPTkemcvNeo was generated by inserting a 582 bp blunt EcoRI, MscI fragment from pBS-ECAT (Jang *et al.*, 1989) into a pBr322 based pPTk+NeopA clone. The resulting vector was modified by exchanging the sequences between the HindIII site in the IRES sequence and the NcoI site at the 5' end of the neo coding sequences for a fragment from the 3' end of the EMCV IRES with the sequences around the translation start site modified to an NcoI site thus placing the ATG in the neo gene onto the starting ATG from the EMCV IRES. The EMCV IRES with a NcoI site on the ATG has been made by exchanging the 3' end of an EMCV IRES clone in pUC119 for a pcr fragment generated with the following primers: 5'-CCCAGTGCCACGTTGTGAGTTGG-3' and 5'-GCGGATCCGGCCATGGTATCATCGTGTTTTTC-3'.

To test the efficiency of translation of both coding regions in the different bicistronic constructs Rat-2 fibroblasts were cotransfected as described above with pRSVLuc and one of the following bicistronic constructs: pPTk+NeopA/IF, pPTk-NeopA/OF, pPTkpolioNeo, pPTkemcvNeo, pPTkpA or pPNeopA (Figure 6). Forty eight hrs after transfection half of the cells was used to measure luciferase activity and the other half was partly plated in different dilutions in medium containing HAT or G418, and partly pooled and subjected to G418 selection 1.4 mg/ml (bicistronic constructs and pPNeopA) or HAT selection (pPTkpA). In three independent cotransfection experiments the number of colonies formed with G418 selection was calculated from the different dilutions that were plated and corrected for differences in transfection efficiency in two ways: 1) by making use of the luciferase activity measured in each of the transfections (Figure 6A) and 2) by making use of the number of colonies formed under HAT selection (Figure 6B). In the latter case the assumption is made that the differences in intercistronic sequences do not influence the translation of the tk gene. As is evident from the figures 6A and B in both cases there are no significant differences in the efficiency by which neo^r colonies are formed after transfection of the bicistronic constructs. The monocistronic construct pPNeopA is, however, about 4x as efficient compared to the bicistronic constructs.

The transfected Rat-2 cells that were pooled and selected with G418 can be used to analyse co-expression of the tk gene by monitoring cell kill after growth in medium supplemented with ganciclovir (GCV). Hereto cells from the different pools were plated in quadruple in 96 well plates at 800 cells/well and grown for 4 days in the presence of 0, 5, 10 or 25 µM GCV. The number of GCV resistant, viable cells was then determined colorimetrically by means of MTS staining (Promega). The results are presented in table 2 below. The absorbance at 490 nm in the wells containing no GCV is set to 100 %.

Cell pool	Canciclovir concentration (mM)			
	0 µM	5 µM	10 µM	25 µM
pPTk+NeopA/IF	100 ± 11.1	3.4 ± 1.0	2.6 ± 2.0	2.8 ± 2.1
pPTk-NeopA/OF	100 ± 15.7	4.2 ± 2.1	4.5 ± 2.5	5.1 ± 2.9
pPTkpolioNeo	100 ± 13.8	2.3 ± 2.0	1.8 ± 1.4	2.3 ± 1.6
pPTkemcvNeo	100 ± 15.2	0.2 ± 0.4	0.2 ± 0.2	0
pPTkpA	100 ± 19.6	0.2 ± 0.5	1.0 ± 2.0	0
pPNeopA	100 + 12.3	96 ± 3.0	102 ± 25	87 + 17

Table 2: % of surviving cells grown in medium with different concentrations GCV relative to cells grown in normal medium

Cell kill is almost complete at 5 µM GCV, a concentration that can be reached in the blood of a patient without any effect on normal cells. A similar experimental set up was used to test lower concentrations of GCV ranging from 0.125 to 1.0 µM. As is shown in table 3 below, pools of cells containing an IRES sequence to express the neo^r gene in a bicistronic transcription unit have a slightly lower LC_{50} (GCV concentration at which 50% of the cells are killed) as compared to the cell pools generated with the bicistronic constructs containing an intercistronic linker. In addition, complete cell kill is reached at slightly lower concentrations of GCV in case of the IRES containing constructs.

Cell pool	Ganciclovir concentration (µM)					
	0	0.125	0.25	0.5	0.75	1.0
pPTk+NeopA/IF	100 ± 7.1	90.8 ± 6.9	48.8 ± 1.7	17.8 ± 2.3	13.1 ± 2.4	9.7 ± 5.3
pPTk-NeopA/OF	100 ± 6.3	80.5 ± 10.9	45.6 ± 8.8	18.7 ± 2.1	11.4 ± 3.7	9.9 ± 1.4
pPTkpolioNeo	100 ± 8.2	62.9 ± 3.5	27.4 ± 1.3	8.5 ± 0.4	3.1 ± 0.7	1.4 ± 1.3
pPTkemcvNeo	100 ± 11.5	67.2 ± 7.0	34.1 ± 2.2	9.7 ± 1.0	2.6 ± 1.9	0.3 ± 0.3
pPTkpA	100 ± 10.8	44.9 ± 10.2	21.4 ± 1.8	8.1 ± 2.7	8.7 ± 3.6	6.6 ± 2.3

Table 3: % of surviving cells grown in medium with different concentrations GCV relative to cells grown in normal medium

Mixing of HSV-tk$^+$ cells with HSV-tk$^-$ cells showed that, at the cell densities used in these experiments, there is no influence of the so called 'bystander effect', proving that cell kill is due to endogenous expression of HSV-tk and not due to transfer of the toxic substance to tk$^-$ cells (results not shown).

Above experiments demonstrate that intercistronic linkers can be used in a bicistronic transcription unit in stead of IRES sequences without losing functional expression of either gene in the bicistron. Synthetic intercistronic linkers are preferred over virus-derived IRES sequences because they are shorter in size and do not form strong secondary structures in the RNA.

Example 2: Retroviral vectors useful for transfer of the human multidrug resistance-1 gene

Multidrug resistance may result from synthesis of a multidrug transporter (P-glycoprotein) encoded by the 'multidrug resistance (*MDR1*)' gene. It is possible to confer a multidrug-resistant phenotype to drug-sensitive cells by transfection and subsequent expression of the *MDR1* gene. An attractive approach therefore would be to introduce the *MDR1* gene into haemopoietic stem cells (HSC), with the objective to protect patients from drug-induced myelotoxicity. Alternatively, the *MDR1* vector could be used to introduce yet another gene of interest (e.g. Glucocerebrosidase gene or HIV inhibiting genes/sequences) allowing selection *in vivo*, using cytotoxic drugs efficiently pumped out of the cell by P-glycoprotein. Successful circumvention of myelosuppression by transduction of the *MDR1* gene in bone marrow cells is dependent on an efficient gene transfer system. Currently, retrovirus-mediated gene transfer is the only technique that allows efficient and stable gene transfer into HSC.

An optimal retroviral construct for introduction of the human *MDR1* gene in haemopoietic cells has besides the

properties of the basic pLecΔMo construct the following additional properties:

- Only the full length coding DNA sequence of the *MDR1* gene is inserted to avoid negative influence of non-coding flanking nucleotide sequences on gene expression and to allow maximal space for additional gene(s) of interest.
- The wild-type *MDR1* is utilised instead of mutant forms with altered substrate specificity (Choi *et al.*, 1988) as mutant *MDR1* P-glycoproteins may result *in vivo* in an immunogenic response to transduced cells.

Construction of pIGmdr1-G

To facilitate cloning of the *MDR1* gene, the high-copy number plasmid backbone in pLTKkoz was replaced by a low-copy number plasmid by subcloning of the NheI fragment of pLTKkoz in pZIPΔMo+PyF101(N-). The resulting construct was named LTK-ΔMo. The wild-type human *MDR1* cDNA (van der Bliek *et al.*, 1988) was inserted in LTK-ΔMo by ligation of 3 fragments:

Fragment 1: NcoI-BamHI fragment of LTK-ΔMo.

Fragment 2: NcoI-EcoRI MDR1 fragment.

This 1178 bp fragment was generated by PCR using Pfu DNA polymerase. Two primers were used: 'mdr5'(thio)' (5'-CCTCTAGACCATGGATCTTGAAGGGGACCGCAA
TGGAGGA-3') spanning the start-codon of *MDR1*, in which a cytosine was placed before the ATG start-codon, thereby creating a NcoI site at this position. The second primer (4728A: 5'-CCAACCAGGGCCACCGTCTGCCCA-3') is positioned 3' of the EcoRI site in *MDR1*. This fragment was first digested with EcoRI and subsequently partially digested with NcoI. The 1.2 kbp fragment was isolated from an agarose gel.

Fragment 3: EcoRI-BamHI 3' MDR1 fragment.

This fragment was isolated from subclone K1. K1 resulted from a previous 4-part ligation to generate another retroviral vector expressing the *MDR1* gene which was performed as follows. The wild-type *MDR1* gene, inserted in pJ3ΩMDR1.1 is described in Schinkel *et al.*, 1991. A 3.8 kbp DraI-HhaI fragment was isolated from this construct. This fragment was ligated to two double strand linkers: a 5'-linker (5'-CTCTGAGCTCCCATGGATCTTGAAGGGGACCGCAATGGAGGAGCAAAGAAGAAGAACTTTTT

TAAATCTC-3') which was cut with NcoI and DraI and isolated from a agarose gel and a 3'-linker (5'-CAGGCTGGAACAAAGCGCCAGTGAGGATCCTCTCT-3') which was cut with HhaI and BamHI and also isolated from a agarose gel (bold sequence indicates fragment to be inserted). After ligation, the product was recut with BamHI and ligated to a NcoI-BamHI retroviral fragment. After transformation in competent DH5α cells, a clone was isolated, K1, which had a correctly inserted 3' HhaI-BamHI MDR1 fragment as confirmed by sequence analysis. However, the 5' oligonucleotide was not inserted. Therefore, K1 has a BamHI-site directly positioned after the TGA stop-codon which could be used to isolate the EcoRI-BamHI MDR1 fragment.

Ligation of the three fragments 1,2 and 3 resulted in pIGmdr1-G and was used to generate *MDR1* producer cell lines.

Generation of the IGmdr1-G retroviral producer cell line: IGvp010

IGmdr1-G was transfected in the PA317 packaging cell line (obtained from the American Type Culture Collection: ATCC No. CRL 9078). PA317 cells were selected in HAT medium to select for cells that retain the packaging function. The cells were grown in HT medium for 4 days to dilute residual amethopterin. $6x10^5$ cells were transfected with 6 µg IGmdr1-G DNA using LipofectAMINE as reagent. The following day cells were trypsinized and $1.0x10^6$ cells were seeded per 75 cm^2 dish. The next day 70 nM of vincristine was added. Medium with vincristine was refreshed every 3 days. Two weeks after trypsinization vincristine resistant colonies were trypsinized, pooled, and further cultured. The resulting cell line was called IGvp010.

Transduction and expression studies with IGmdr1-G

a) Test for the presence of helpervirus

IGvp010 was proven to be free of replication competent retroviruses (RCR). This was determined by co-cultivation of $5x10^6$ cells for 5 passages with *Mus dunni* cells which permits amplification of RCR by the feline (PG-4) S+L- focus assay.

b) Expression of IGmdr1-G in a human drug-sensitive A2780 cell line.

To simulate bulk transduction conditions that are employed for haemopoeitic target cells we tested supernatant harvested from the IGvp010 producer cell line on a P-glycoprotein negative human ovarian tumour cell line (A2780). After a two-hour transduction and a 48 hour culture period, it was possible to accurately determine the proportion of transduced cells by their ability to exclude the fluorescent dye Rh-123 which is an efficient substrate of the P-glycoprotein. Under these conditions the IGvp010 supernatant yielded 29.37 % Rh-123^{dull} cells (corrected for background activity, Figure 7).

c) Transduction of mobilised human peripheral blood progenitor cells (PBPC).

$CD34^+$ selected PBPC were transduced over 96 hours with IGvp010 supernatant at a cell concentration of 1 x 10^6/ml in the presence of human interleukin-3. IGvp010 supernatant was refreshed every 24 hours. Protamine sulphate (4 µg/ml) was added with every supernatant change. *MDR1*-transduced and mock-transduced PBPC were plated in duplicate at 5 x 10^3/ml in 1 ml methylcellulose medium in the presence of IL-3 and GM-CSF. Screening for *MDR1* overexpressing progenitor cells was performed with vincristine which is an efficient substrate for the P-glycoprotein. Freshly thawed vincristine was added to the dishes in increasing amounts. At all concentrations, cells were plated in duplicate. Colony forming units (CFU-C) were scored after 14 days. *MDR1*-transduced and mock-transduced were compared by dividing the number of colonies in the dishes with vincristine by the number of colonies in the dishes without vincristine. Figure 8 shows the result (error bars give the minimum and maximum value obtained). At a dose of 20 nM, 47% of the IGmdr1-G colonies survived compared to 3% of the mock infected cells. A dose of 30 nM killed all colonies in the control group, while still 9% of the *MDR1*-infected colonies survived. This experiment not only clearly demonstrates that IGmdr1-G efficiently infects haemopoietic cells, but also has a high expression level of the inserted gene in haemopoietic precursor cells.

d) Transduction of normal human bone marrow cells.

CD34+ selected normal human bone marrow cells were transduced as described for PBPC. After transduction, cells were seeded for CFU-C formation in the presence of increasing amounts of vincristine. Individual CFU-Cs were picked and DNA isolated from the colonies was subjected to a provirus-specific PCR. Seven independent experiments demonstrated that 8±9 percent of the CFU-C was transduced with the IGmdr1-G retrovirus. From one experiment, also vincristine resistant colonies were analyzed. This experiment showed that the percentage PCR+ CFU-C increased from 30% without vincristine to 44% (20 nM), 71% (30 nM) and 100% at 40 nM drug. This experiment clearly demonstrates that in vitro selection of transduced hemopoietic progenitor cells at increasing doses of cytostatic drug actually occurs.

In accordance with results presented by Sorentino *et al*. (1993), we detected aberrant splicing of the RNA derived from the *MDR1* cDNA inserted in our vectors. An (obvious) improvement would be the modification of cryptic splice sites without altering amino acid coding sequences.

Example 3: Gene therapy for AIDS/*in vivo* selection of transduced HSC

Infection of CD4+ T-cells by the human immunodeficiency virus (HIV) is the first and causative event in the development of AIDS. As a member of the large family of retroviridae HIV has an RNA genome and a life-cycle like other retroviruses. Spread of the virus is depending on infection, reverse transcription, integration, transcription and packaging of the viral genome. Gene therapy strategies have been developed that interfere with the life cycle of the retrovirus using so called genetic antivirals like e.g. intracellular antibodies, ribozymes, antisense molecules or decoys (reviewed in Gilboa and Smith, 1994). These molecules have to be delivered to the cells primarily susceptible to infection by HIV i.e. CD4+ T-cells and monocytes/macrophages in the blood. Protection of these cells to HIV infection may limit or even prevent the spread of the virus and limit the pathogenic effect of the virus onto the immune

system. Ideally, haemopoietic stem cells are the targets for such protective therapy as they will provide the patient with a continuous source of protected T-cells. However, the stable infection of HSC may not be very efficient and following transplantation of the transduced cells a multitude of non-transduced endogenous stem cells will continue to generate mature cells resulting in many unprotected cells in the peripheral blood and thus facilitating replication of HIV. Therefore, the constructs that we describe here and that are designed to express genetic antivirals in HSC and descendents thereof, are all based on the pIGmdr1-G retroviral construct (see example 2). In this construct the human *MDR-1* gene allows for selection of transduced stem cells *in vivo*.

<u>Construction of pIGmdr1-G/HIVasTAR and pIGmdr1-G/HIVasTAR*gag* and similar vectors</u>

As an example of the construction of recombinant retroviral vectors specially designed to deliver anti-HIV-1 molecules to the HSC and their descendants, we describe recombinant retroviral constructs generating antisense RNAs directed to the 5' end of HIV-1. The use of the polymerase III-dependent adenoviral VA1 promoter (Fowlkes and Shenk, 1980) ensures high levels of expression of short inserted sequences.

Firstly, a subclone from pIGmdr1-G was generated by digestion with BamHI and religation of vector sequences. This clone, pIGmdrΔBamHI was used to introduce the Adenoviral VA1 gene and promoter sequences that were obtained by amplification of Ad5 sequences with the primers: 5'-CCTGCTAGCTCTAGACCGTGCAAAA-3' and 5'-AAAGCTAGCAAAAAAGCGGCCGCGGGGCTCGAACCCCGGTCGTCC-3'. Digestion of the pcr product with NheI allowed for cloning into either the unique AvrII or NheI site of pIGmdrΔBamHI. Clones were selected that contained the VA1 promoter in either orientation. The unique NotI site that was introduced into the VA1 gene during the pcr amplification, then served as an insertion site for HIV-1 sequences. These were obtained by amplification of HIV-1 sequences in the pBRU2 vector (XbaI/ClaI fragment from pLAI/pBru from B.Klaver, AMC A' dam) using the pcr primers: 5' TAR 5'-AATCGCGGCCGCGTCTCTCTGGTTAGAC-3' with 3' TAR 5'-AATCGCGGCCGCGGTTCCCTAGCTAGCC-3' to amplify the TAR loop from

+1 to+57 (pIGmdr1-G/HIV-TAR) and with 3' *gag* 5'-AATCGCGGCCGCTCTCGCACCCAT-3' to amplify the 5' end up to the *gag* start codon from +1 to +348 (pIGmdr1-G/HIV-*gag*). The pcr fragments were digested with NotI and cloned into the pIGmdrΔBamHI/VA constructs. Depending on the orientation of inserted HIV sequences with respect to the internal WA1 promoter, the constructs pIGmdr1-G/HIVasTAR, /HIVsTAR, pIGmdr1-G/HIVasTAR*gag* or /HIVsTAR*gag* were generated (where s=sense and as=antisense).

In addition, all constructs were derived in two orientations: one in which the VA1 promoter is driving transcription in the same direction as the viral LTR and one in the reversed direction.

Example 4: Retroviral vectors for gene therapy of Gaucher disease and *in vivo* marking studies

Retroviral constructs for the treatment of Gaucher disease should be based on vectors working favourably in the haematopoietic system particular following stem cell gene tranfer. The construction of IGGC therefor carries the glucocerebrosidase (GC) sequence in the retroviral back bone as disclosed in this invention.

Moreover, the construction of IG-GC retroviral vectors that differ in the length of the inserted Glucocerebrosidase (GC) cDNA (described below) was undertaken in order to perform *in vivo* gene marking studies. The difference in length of the inserted GC cDNA's allows for the discrimination between multiple retroviruses after ex vivo infection and reinfusion of the infected graft into the same animal or human. To optimize gene delivery to $CD34^-$ primitive progenitor cells these vectors can be used to study transduction efficiency differences between viruses produced by different packaging cell lines. In addition, different transduction protocols i.e. different growth factors and the role of virus titers can be studied. Compared to single vector gene marking studies, gene marking with multiple, distinguishable vectors enables one to rapidly assess the role of crucial parameters in determining transduction efficiency of $CD34^+$ primitive progenitor cells. Furthermore, an additional advantage of the use of therapeutic

cDNA's, such as hGC, in gene marking studies instead of cDNA's encoding foreign proteins, for instance Neo^r (Brenner *et al.*, 1993), is the absence of unwanted host immune responses against the expressed foreign protein.

Construction of retroviral Glucocerebrosidase vectors, IG-GC

The complete cDNA sequence (1888 bp) of the human placental glucocerebrosidase was digested to completion by XhoI and separated from the 7549 bp pGB125 backbone (Genzyme cooperation) by agarose gel electrophoresis. The DNA fragment was electroeluted from the agarose and purified by phenol/chloroform/ isoamylalcohol extraction.

The pLec plasmid (5773 bp) was linearized by XhoI digestion. The XhoI digested DNA was treated with calf intestinal phosphatase (CIAP) and subjected to agarose gel electrophoresis. The linearized DNA fragment was excised and purified. The isolated XhoI hGC cDNA fragment of 1888 bp was ligated to the dephosphorylated 5773 kb XhoI DNA fragment of pLec using T4-DNA ligase. The resulting 7661 bp retroviral vector is designated IG-GC-1 (Figure 9). To construct retroviral vector IG-GC-2, the IG-GC-1 vector was digested with NheI. The resulting 3431 bp NheI DNA fragment was ligated to the linearized and dephosphorylated 4375 kb NheI DNA fragment of pSK/ZipΔMo+PyF101 using T4-DNA ligase. The resulting 7806 bp retroviral DNA construct is designated IG-GC-2 (Figure 10).

In addition to IG-GC-1 and IG-GC-2, two variants were constructed designated IG-GC-3 and IG-GC-4. These variants are identical to IG-GC-1 and IG-GC-2 respectively except for the 3'-untranslated region of the hGC coding sequence. From this region a 160 bp fragment (from nt. 1728 to nt. 1888) was deleted using PCR. Construction of IG-GC-3 was done as shown in figure 11. Briefly, two oligonucleotides were synthesized, GCo3 with sequence 5'-CGGGATCCTAGAGGGGAAAGTGAG-3' and GCo4 with sequence 5'-CAGCCCATGTTCTACCAC-3'. GCo3 contains a BamHI restriction site. These two oligonucleotides were used to amplify a 420 bp DNA fragment using IG-GC-2 plasmid DNA as template. The 420 bp PCR fragment was digested with BamHI and the 220 bp PCR fragment was isolated and ligated to IG-GC-1

DNA that was linearized with BamHI (Figure 11). The resulting vector, designated IG-GC-3, now contains a human GC cDNA which lacks 160 bp in the 3' noncoding region (Figure 12). To construct IG-GC-4, IG-GC-3 was digested with NheI and a 3231 bp DNA fragment was isolated. This fragment, which includes the hGC cDNA sequence was cloned into the NheI linearized and dephosphorylated pSK/ZipΔMo+PyF101 4375 kb DNA fragment (Figure 13). The resulting 7606 bp retroviral vector is designated IG-GC-4.

Generation of IG-GC-2 and IG-GC-4 recombinant retrovirus producer cells

IG-GC-2 and IG-GC-4 plasmid DNAs were introduced into ecotropic retroviral packaging cell lines GP + E86 (Markowitz *et al.*, 1988) and ψ-CRE (Danos *et al.*, 1988) respectively. Selection of transfected cells was achieved by cotransfection of expression plasmids pSV2neo (Southern and Berg, 1982) with ICG-GC-2 and pPGKneo (R. Vogels, see example 1) with IG-GC4 at a ratio of 1 : 10. G418 resistant cell pools GP2b and ψ4c were generated. Ecotropic IG-GC-2 and IG-GC-4 virus was produced by growing confluent layers of ecotropic producer cells in fresh medium at 32 °C for 24 hours. Virus containing supernatants were collected, passed through a 0.45 µm filter, aliquoted and immediately frozen in liquid nitrogen followed by storage at -80 °C.

To test whether the G418 resistant ecotropic cell pools also express the human GC protein, cell lysates were made and the GC enzyme activity level was measured using an artificial GC substrate (fluorescence: 4-Mu-ß-glucoside or colorimetric: PNP-ß-glucoside). Cell lysates were made from untransfected GP + E86/ψ-CRE cells, transfected GP + 86/ψ-CRE cells, and 3T3 mouse fibroblast cells infected with the GP2b/ψ4c virus supernatant. The results of these measurements showed that the transfected ecotropic GP + E86 /ψ-CRE cells and the infected 3T3 cells had 1.8-2.0 times elevated GC activity levels compared to the non-transfected packaging cells (Figure 14A). A Western blot of cell pools A, B and C (3 independent IG-GC-2 transfections) using the human GC specific monoclonal antibody 8E4 (Aerts *et al.*, 1985) showed that the 59 kDa hGC protein is expressed in the GP + E86 packaging cell line (Figure 14B).

To show that the increased GC enzyme activity levels after infection of cells with ecotropic virus is caused by expression of the human GC protein, ecotropic virus obtained from the GP2b pool was used to multiply infect amphotropic PA317 cells. Cell lysates of the infected PA317 cells were made and the GC activity level was measured. It was shown that the infected cell pool had approximately 1.8-2.0 times elevated GC activity levels compared to the non-infected packaging cells (data not shown). A Western blot of the cell lysates of these infected PA317 cell pools (B1 and B2), using the human GC specific antibody 8E4 (Aerts *et al.*, 1985), clearly shows that ecotropic virus, carrying the IG-GC-2 vector, transfers the human GC protein at significant levels (Figure 15).

Using the same ecotropic IG-GC2/IG-GC4 virus supernatant, Gibbon ape leukaemia virus (GALV) packaging cells (PG13) were infected. In the generated PG pools hGC activity was measured as described above and again proved to be 2 to 3 times elevated compared to parental PG13 cells (data not shown).

Next, producer clones were isolated from these pools. In order to achieve this two rounds of limiting dilution (<1 cell/well) were performed in 96-well microtiter plates. Virus production from these clones was initially tested by measuring hGC activity in NIH/3T3 cells (PA317 derived) or Rat-2 fibroblasts (PG13 derived) after incubation with the cell culture supernatant. Clones giving the highest increase in hGC activity were selected and designated PA2 (PA3 17/IG-GC2), PA4 (PA3 17/IG-GC4), PG2 (PG13/IG-GC2), and PG4 (PG13/IG-GC4). These cell lines are deposited at the ECACC under Nos: PG4 - 96050256, PG2 - 96050257, PA4 - 96050258 and PA2 - 96050259 according to the Budapest treaty.

<u>Efficacy testing of the isolated retrovirus producer cell clones</u>

To characterise endogenous hGC expression lysates were prepared from the producer cells. In addition, lysates were prepared from Gaucher type II fibroblasts infected with PA2, PA4, PG2, and PG4 virus supernatants to compare virus titer. A Western analysis of these protein lysates indicates a

correlation between endogenous hGC expression in the producer clones and virus titer i.e. the producers with high endogenous hGC levels yield high titers (Fig. 16).

The virus titer of the PA2 producer was determined by incubating 10^5 NIH/3T3 cells (6-well plates) with 1 ml of PA2 virus supernatant over a 48 hour period. Subsequently, the infected NIH/3T3 cells were subjected to one round of limiting dilution (>1 cell/well) and 50 individual clones were expanded in order to measure hGC activity and to isolate genomic DNA for copy number determination. All of these 50 NIH/3T3 clones expressed hGC. Southern analysis revealed that in each transduced individual NIH/3T3 clone at least three separate integrations took place. From these results it was concluded that the titer of the PA2 producer is at least 3 x 10^5 functional virus particles/ml. (Fig. 17).

To investigate whether the biochemical phenotype in Gaucher type I and type II primary fibroblasts could be reversed, these cells and two normal human primary fibroblast cells were incubated with virus supernatant of the PA2, PA4, PG2, and PG4 producer cell clones (Fig 18). The data show that a single infection with either PA2 or PG2 virus supernatant is sufficient to augment hGC activity levels in the Gaucher type I and II infected cell pools to those comparable to uninfected normal human fibroblasts. Thus, correction of the biochemical Gaucher phenotype is accomplished by infection with these recombinant viruses carrying the IG-GC constructs. The data also show that a single incubation with either PA4 or PG4 supernatant on Gaucher type I and II fibroblasts increases hGC activity levels to 50-70% compared to normal.

Transduction of human $CD34^-$ cells

CD34+ cells were isolated from total bone marrow harvested from a Gaucher type I patient. These $CD34^+$ cells were seeded at a concentration of 10^5 cells in 24-well plates in 400 ml virus supernatant supplemented with 4 mg/ml protamine sulphate, IL-3, and pen/strep. Daily, for four executive days, the virus supernatant was refreshed. After this transduction period 2.5 x 10^4 cells were seeded in a liquid culture assay (medium containing IL-3, IL-6, SCF,

GM-CSF, and G-CSF). After a 10 day incubation at 37°C/10% CO_2 the cells were harvested and counted. The number of cells obtained after this 10 day period normally was between 5×10^5-1×10^6 showing a proliferative capacity factor of 20 to 40 times. Nine-tenth of the cells was used to measure elevation of hGC activity. One-tenth of the cells was pelleted and lysed for PCR. Figure 19 shows the elevation of hGC activity in the differentiated Gaucher type I cells derived from the liquid culture after transduction with either IG-GC2/IG-GC4 or MDR virus. PCR and subsequent Southern analysis of the cells derived from the liquid culture shows that the provirus is present in the IG-GC2/IG-GC4 transduced cells (fig. 20).

Besides the liquid culture, $CD34^+$ cells were also seeded for a colony forming unit (CFU) assay at a concentration of $5x10^3$/ml in semi-solid methylcellulose. After a 14 day period in an incubator set at 37°C/10% C02 approx. 100 individual colonies were picked to investigate the transduction efficiency of the virus supernatants on these primitive human cells. By means of PCR it could be shown that with either PA2 or PG2 recombinant virus approx. 40-50% of all CFUs contained the provirus. Infection of this cell type with PA4 proved to be less efficient with approximately 10% infected CFUs (data not shown).

From these data it can be concluded that the IG-derived viruses are potent gene delivery vehicles capable of correcting the biochemical Gaucher phenotype in primary fibroblasts and $CD34^+$ hemopoietic Gaucher cells.

<u>Construction of retroviral Glucocerebrosidase-dihydrofolate reductase dual vectors</u>

Low transduction efficiency of the human hemopoietic stem cells poses a serious limitation with respect to successful gene therapy for diseases such as Gaucher disease. A potential strategy to circumvent this problem is to use retroviral vectors carrying a therapeutic cDNA and a dominant selectable marker. Such retroviral vectors make it possible to select transduced cells *in vivo*. For this reason we constructed bicistronic retroviral constructs carrying the hGC cDNA and

the methotrexate resistant cDNA of human dihydrofolate reductase (hDHFR).

The human wild type DHFR was amplified with Pwo enzyme from single stranded cDNA synthesized from mRNA of human liver. The 5'oligonucleotides DHFR1 (5'cccaagcttcccgggctgcagcgccaccatggttggttcgctaaactg-3') and the 3' oligonucleotide DHFR2 (5'ccatcgatctcgagtcattcttctcatatacttcaaa-3') yielded the expected DNA fragment of 550 bp. To obtain a methotrexate resistant version of hDHFR a point mutation, Phe32Ser, was introduced in the wild type hDHFR cDNA. Therefore, a 120 bp 5' part of hDHFR was amplified with oligonucleotides DHFR1 and DHFR4 (5'-gaaatatctagattcattcctg-3') which carries the desired mutation, full length hDHFR PCR product as template, and Pwo enzyme. In an additional PCR reaction a 430 bp 3' part of hDHFR was amplified with DHFR2 and DHFR3 (5'-caggaatgaatctagatatttc-3') which is the reverse complement of DHFR4. Both 5'-part and 3'-part were denatured and annealed to each other and subsequently amplified with DHFR1 and 2 to reassemble the 550 bp full length hDHFR (Fig.21). The presence of the desired mutation was analyzed by digestion with EcoRI which cuts in the wild type hDHFR but not in the Phe32Ser mutant.

To delete the entire 3'-noncoding domain of hGC. IG-GC2 plasmid was utilized for the amplification of a 300 bp hGC DNA fragment with oligonucleotides GCol1 (5'-gatcgagggatgcagtac-3') and oligonucleotide GCol4 (5'-tgtggcgtcgccagtgaggatcctctagaagcttggg-3'). Oligonucleotide GCol4 contains the stopcodon of hGC. Downstream of oligonucleotide GCol1 a unique SalI site is present and oligonucleotide GCol4 contains a HindIII site. For cloning purposes, the wildtype and Phe32Ser mutant hDHFR PCR fragments were digested with HindIII (present in DHFR1) and ClaI (present in DHFR2). The 300 bp 3'-hGC PCR fragment was digested with SalI and HindIII. These two DNA fragments were ligated in a three point ligation reaction into a SalI, ClaI digested IG-GC1 construct (Fig. 22). The resulting bicistronic vectors were coded IG-GC5 (wildtype hDHFR) and IG-GC6 (Phe32Ser hDHFR). In these vectors the hGC coding region is

separated from the hDHFR coding region by a 36 bp intercistronic linker enabling translation of both proteins from one single mRNA. To obtain bicistronic retroviral vectors containing the mutant polyoma enhancer PyF101 in the 3'-LTR, the NheI fragments were isolated and cloned into pSK/ZipDMo+PyF101 (IG-GC7 and IG-GC8 respectivily). These bicistronic retroviral vectors can be transfected into retroviral packaging cell lines to generate viruses that upon infection of target cells render these cells resistant to methotrexate, a potent cytotoxic drug which inhibits DNA synthesis by depleting the pool of pyrimidines.

Example 5: Introduction of Locus Control Region sequences of the human CD2 gene in the recombinant retroviral vector pLgAL(ΔMo+PyF101).

For this example the retroviral vector construct pLgAL(ΔMo+PyF101) (Van Beusechem *et al.*, 1990) was used, wherein A represents the human cDNA gene encoding adenosine deaminase (hADA), which is further referred to as "the vector". Additionally, the Locus Control Region (LCR) sequence from the 3'regionof the human CD2 gene (Lang *et al.*, 1988) was used, which is further referred to as "CD2-LCR". In the CD2-LCR a 2076 nt HindIII fragment (nt 2-2077) has been identified which in transgenic mice exerts all the characteristic features of the CD2-LCR on the CD2 promotor as well as on heterologeous promoters (Lang *et al.*, 1988, Lang *et al.*, 1991). Within this fragment lies a 880 nt AflIII fragment (nt 433-1314), of which it has been shown in human T-cell lines *in vitro* that it act as a CD2-LCR (Lake *et al.*, 1990). In the vector the HindIII CD2-LCR fragment, further referred to as "L2", or the AflIII CD2-LCR fragment, further referred to as "L0.8", was cloned. Thereto the L2 and L0.8 fragments were isolated from the construct GSE1502 (D. Kiousis, MRC) and provided with a blunt end with Klenow-polymerase. The vector was digested with ClaI (nt 7675 of Mo-MuLV, in *env*) or NheI (nt 7846 of Mo-MuLV, in the 3'LTR) and also provided with a blunt end. The fragments L2 or L0.8 were cloned into the ClaI site (resulting constructs are further referred to as "CL2" or "CL0.8"), or into the NheI site (resulting constructs are

further referred to as "NL2" or "NL0.8"). They were cloned in the normal 5'-->3' orientation of the CD2-LCR (forward, further referred to as "F") as well as in the 3'-->5' orientation (reverse, further referred to as "R"). In this way 8 different novel retroviral constructs were made, referred to as "CL2F", "CL2R", "CL0.8F", "CL0.8R", "NL2F", "NL2R", "NL0.8F", and "NL0.8R" (Figure 2).

The 8 new constructs were packaged into recombinant retroviruses. Thereto 20 µg DNA of the constructs was transfected into the ecotropic packaging cell line GP+E-86 (Markowitz *et al.*, 1988), using the method as described by Chen and Okayama (1977). Prior to the transfection the GP+E-86 cells were cultured in a medium containing 15 µg/ml hypoxanthine, 250 µg/ml xanhine, and 25 µg/ml mycophenolic acid, in order to select for retaining the DNA sequences which are responsible for the production of viral proteins. Transfectants producing a functional hADA enzym were isolated through a selective culture in medium containing 4 µM xylofuranosyl-adenine (Xyl-A) and 10 nM deoxycoformycin (dCF) (Kaufman *et al.*, 1986). Culture supernatant of Xyl-A/dCF-resistant transfectants was, after filtration through a filter with a pore size of 0.45 µm, used to transduce the amphotropic packaging cell line GP+*env*Am12 (Markowitz *et al*, 1988) with the ecotropic recombinant retroviruses present in that culture supernatant. The amphotropic packaging cells were selected for retaining the DNA sequences encoding viral proteins prior to use (as described for GP+E-86 cells, with the addition of 200 µg/ml hygromycine B) and preincubated with 4 µg/ml polybrene to promote retrovirus transduction. GP+*env* Am12 cells producing a functional hADA enzym were isolated through Xyl-A/dCF-selection as described above. Individual hADA-positive GP+*env*Am12 clones were isolated and expanded. Before the hADA-positive GP+*env*Am12 clones were characterized for integrity of the integrated recombinant retroviruses and the production of amphotropic recombinant retroviruses it was first verified that all clones were derived from individual transductions with recombinant retroviruses. Hereto chromosomal DNA from the clones was digested with BglIII (which cuts once in the construct, within the hADA gene) and

hybridised with an hADA probe (551 nt BglIII-SstI fragment of pAMG1 (Valerio *et al.*, 1985)) in a Southern analysis. Herewith 3'junction fragments with a length which depends on the insertion site in the genome are identified. All clones were shown to have one single insertion of one of the recombinant retroviruses. Clones having junction fragments of the same length were excluded from further analysis. Thereafter all remaining clones were tested for the correct structure of the integrated recombinant retroviral construct through Southern analysis. Hereto the chromosomal DNA of the clones was digested with KpnI (cuts once in both retroviral LTRs, resulting in fragments which hybridise with de hADA probe of 3.5 kb for the vector and 5.5 kb and 4.3 kb for constructs having L2 and L0.8 insertions, respectively). The result of this analysis is shown in Table 2. When the L2 fragment was cloned in the ClaI site the fragment length in all analysed clones was correct, independent of the orientation of the insertion. Cloning in the NheI site, however, resulted in instability of the resulting constructs. This result was most serious after insertion in the forward orientation. Insertion of the L0.8 fragment resulted in stable recombinant retrovirus constructs in most cases, except after the cloning into the ClaI site in the reverse orientation. All clones packaged recombinant retroviruses; even the clones which harboured damaged retrovirus insertions. The different insertions had no significant effect on the titers with which viruses were produced (Table 4). A number of clones harbouring truncated retrovirus insertions was subjected to a more thorough analysis. Three independent NL2R clones were all shown to have a 2 kb deletion in a fragment covering the area from the 3'end of the hADA gene until the 3'end of the L2R insertion. In 7 analysed NL2F clones the retrovirus insertions were shown to have deletions varying from 200 bp to 2 kb in size. In all 7 clones the deletion comprised (a part of) the L2F fragment, in 2 of these clones the deletion extended into the vector (3' of hADA and 5' of L2F in the 3'LTR). The 5 damaged CL0.8 clones had deletions of different lengths in 4 cases and an insertion in one case. These results show that the L2 fragment comprises sequences which influence the stability of the vector

negatively when this fragment is incorporated the LTR of the vector. Therefore, L2 fragments must be placed between the LTRs, whereby it is preferred to use the ClaI site for that purpose. As an alternative for insertion into the LTR the smaller L0.8 fragment can be used.

Table 4: Analysis of GP+*env*Am12 clones harbouring a single copy of a recombinant CD2-LCR comprising retrovirus construct, obtained through infection with ecotropic recombinant retrovirus supernatant.

Recombinant Retrovirus Construct	Stability Retrovirus Structure*	Recombinant Retrovirus Titer [mean (range)]**
CL2F	9/9	9E2 (1E2-2E3)
CL2R	4/4	2E3 (1E3-4E3)
CL0.8F	7/7	9E3 (1E3-2E4)
CL0.8R	7/12	N.A.
NL2F	0/7	2E4 (1E3-1E5)
NL2R	8/10	3E3 (5E1-1E4)
NL0.8F	6/6	2E3 (1E2-6E3)
NL0.8R	4/4	5E3 (2E3-8E3)

* Number of clones having the correct recombinant retrovirus integration / number of independent clones analysed.

** Mean titer of GP+*env*Am12 cells harbouring the vector without the CD2-LCR insertion was 1.5E3. N.A., not analyzed.

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Van Beusechem, V.W., Bart-Baumeister, J.A.K., Hoogerbrugge, P.M. and Valerio, D. Influence of Interleukin-3, Interleukin-6 and Stem Cell Factor on retroviral transduction of rhesus monkey CD34+ haematopoietic progenitor cells measured *in vitro* and *in vivo*., Gene Ther.2 (1995) 1-11.

Van Beveren, C., Coffin, J. and Hughes, S. (1985). Nucleotide sequences complemented with functional and structural analysis, p.766-782 and p.900-911. In R. Weiss, N. Teich, H.Varmus and J. Coffin (Eds.), RNA Tumour Viruses, Vol.2. Cold Spring Harbor Laboratory, Colg Spring Harbor, NY>

Van der Bliek, A.M., Kooiman, P.M., Schneider, C., Borst, P. (1988). Sequence of mdr3 cDNA encoding a human P-glycoprotein. Gene 71, 401-411.

Vincent, A.J.P.E., Vogels, R., Van Someren, G., Esandi, M.C., Noteboom, J.L., Avezaat, C.J.J., Vecht, C., Van Bekkum, D.W., Valerio, D., Bout, A. and Hoogerbrugge, P.M. 1996). Herpes simplex virus thymidine kinase gene therapy for rat malignant brain tumors. Hum.Gene Ther. 7, 197-205.

Weizsaecker, M., Deen, D.F., Rosenblum,M.L., Hoshino, T., Gutin, P.H. and Barker, M. (1981). The 9L rat brain tumour: description and application of an animal model. J. Neurol. 224, 183-192.

Legends to figures

Figure 1. Schematic representation of the cloning strategy used to construct pLec. LTR, long terminal repeat;HIII, HindIII;mcs, multiple cloning site; SD, splice donor site; SA, splice acceptor site; TAG, stop codon; waved lines represent mouse genomic flanking sequences.

Figure 2. Schematic representation of the cloning strategy used to construct pLecΔMo.
x, XbaI and see legend figure 1.

Figure 3. Schematic representation of the cloning strategy used to construct pLTKkoz.
see also legend figure 1.

Figure 4a and b. Schematic representation of the constructs pIGTK and pLTTΔMo. see also legend to figure 1.

Figure 5. Kaplan-Meier survival curves of rats with brain tumour treated with single dosis recombinant retrovirus producer cell lines and subsequent GCV administration. Intracerebral injection of tumour cells at day 0. IG-RV-TK was injected 3 days after tumour cell injection. One group was injected with IG-RV-TK producer cells (n=10)(———). The controls (n=5/ group) were injected with PBS (———), RV-lacZ producer cells (--------) TK+RV- non producer cells (— —), supernatant of IG-RV-TK producer cells (— – —) and RV-IL2 producer cells (— - - —). One group was injected with IG-RV-TK producer cells without subsequent GCV treatment (- - - - - -) . Five days after virus injection 15 mg/kg GCV was administered twice a day for ten days i.p.. IG-RV-TK treated rats lived significantly longer than controls ($p<0.01$; log rank test). One rat died of superficial leptomeningeal tumour (*). This rat is censored in the survival analysis.

Figure 6. Schematic representation of the constructs used to test the intercistronic linkers. TGA, stop codon; E, EcoRI; S, SalI.

Figure 6A. Bar diagram representing the average number of colonies obtained after G418 selection of transfected Rat-2 cells corrected for (arbitrary units of) Luciferase activity.

Figure 6B. Bar diagram representing the average number of colonies obtained after G418 selection of transfected Rat-2 cells corrected for the number of colonies obtained after selection in HAT medium.

Figure 7. Rh-123 exclusion analysis on A2780 cells after transduction with IGmdr1-G virus supernatant. Transduced A2780 cells (A) are compared to mock-infected controls (B).

Figure 8. Vincristine survival of $CD34^+$ peripheral blood cells after transduction with IGmdr1-G virus supernatant. CD34 selected PBPC were transduced with (squares) or without (circles) IGmdr1-G supernatant for 4 days in the presence of IL-3 and 4 µg/ml protamine sulphate. Supernatant was refreshed daily. After transduction, PBSC were seeded for *in vitro* colony formation (GM-CFU) in the presence of increasing amounts of vincristine. Colonies were scored after 14 days. The survival was calculated by dividing the number of GM-CFU in the dishes with drugs by the number of GM-CFU in the dishes without drugs.

Figure 9. Physical map of retroviral construct IG-GC-1. SD, splice donor: SA, splice acceptor. TAG, mutated startcodon of gag coding sequence; LTR, long terminal repeat.

Figure 10. Physical map of retroviral construct IG-GC-2. SD, splice donor; SA, splice acceptor; TAG; mutated startcodon of gag coding sequence; LTR, long terminal repeat.

Figure 11. PCR strategy to delete part of the 3'- untranslated region of the human Glucocerebrosidase cDNA (see text for details).

Figure 12. Physical map of retroviral construct IG-GC-3. SD, splice donor; SA, splice acceptor; TAG, mutated startcodon of gag coding sequencel; LTR, long terminal repeat.

Figure 13. Physical map of retroviral construct IG-GC-4. SD, splice donor; SA, splice acceptor; TAG, mutated startcodon of gag coding sequence; LTR, long terminal repeat.

Figure 14A. Increase in glucocerebrosidase enzyme activity after transfection of retroviral constructs IG-GC-2 and IG-GC-4 in packaging cell lines GP + E 86 and Psi-CRE respectively and after infection of 3T3 cells.

Figure 14B. Western blot with primate specific monoclonal antibody 8E4 (80 mg total protein/lane). A, B, C: Cell lysates prepared from GP + E86 cells after transfection with retroviral construct IG-GC-2. +/-: With or without protease inhibitors. GP: Cell lysate prepared from non-transfected GP + E86 cells.

Figure 15. Western blot with primate specific monoclonal antibody 8E4 (80 mg total protein/lane). B1/B2: Cell lysates prepared from PA317 cells after repeated infection (1, 4, 10 times) with ecotropic GP2b virus (duplicate). GP: cell lysate prepared from GP + E86 cells after transfection with retroviral construct IG-GC-2 as positive control. PA: Cell lysate prepared from non-infected PA317 cells. Cer: 16.5 mg (0.7 units) recombinant glucocerebrosidase (Genzyme Corp.) as positive control.

Figure 16. Western analysis on hGC expression in producer (PA2, PA4, PG2 and PG4) and of the parental cell line PA317 (PA) was loaded on a 10% acrylamide gel. As positive control approx. 1 unit of Cerezyme (Genzyme corp.) was loaded. B) 20µg total protein of each infected Gaucher type II cell pool (PA2, PA4, PG2 and PG4) and of the parental cell line (T-II) was loaded.

Figure 17. DNA analysis of PA2 infected individual 3T3 cell clones. Isolated DNA was digested with EcoRI (unique restriction site just 5' of hGC sequence) and Southern blots were probed with the complete hGC sequence. In the genomic DNA of each individual 3T3 clone a mean of three bands is visible resulting in an estimated functional titer of at least 3×10^5/ml.

Figure 18. hGC-activity assay (PNP-β-Glu) on normal and Gaucher type I and II fibroblasts as well as on type I and II Gaucher fibroblasts infected with virus supernatant. A) Infected Gaucher type I fibroblasts (grey bars) versus non-infected (white bar) and normal (black bar) cells (n=4). B) Infected Gaucher type II fibroblasts (grey bars) versus non-infected (white bar) and normal (black bar) cells (n=4).

Figure 19. hGC-activity assay (PNP-β-Glu) in lysates of infected $CD34^+$liquid culture cells of Gaucher bone marrow (n=4).

Figure 20. PCR and subsequent Southern analysis show the presence of the recombinant provirus in infected $CD34^+$ Gaucher bone marrow cells. Southern blot was probed with a 300 bp BamHI fragment from the 3' end of the hGC gene.

Figure 21. Strategy for the introduction of a point mutation (Phe32Ser) in the human dihydrofolate reductase cDNA.

Figure 22. Schematic drawing of the map of recombinant retroviral vectors IG-GC5 and IG-GC6.

CLAIMS.

1. Vector derived from a retrovirus, comprising a sequence responsible for transcriptional control, including an enhancer,which vector further comprises a site for insertion of at least one gene of interest, a packaging signal, said vector having no superfluous retroviral sequences and no open reading frame encoding at least parts of viral proteins, characterized in that at least one enhancer is an enhancer that is active in undifferentiated cells.

2. A vector according to claim 1, wherein a sequence responsible for transcriptional control is a viral long terminal repeat sequence.

3. A vector according to claim 2 wherein at least one long terminal repeat is of retroviral origin.

4. A vector according to claim 3, wherein at least one long terminal repeat is derived from a Moloney murine leukemia virus.

5. A vector according to anyone of the aforegoing claims, wherein at least one enhancer is the polyoma virus mutant PyF101 enhancer.

6. A vector according to anyone of the aforegoing claims comprising as a site for the insertion of at least one gene of interest a poly cloning site.

7. A vector according to anyone of the aforegoing claims additionally comprising a consensus Kozak sequence at a site where it enhances translation of the gene of interest.

8. A vector according to anyone of the aforegoing claims additionally comprising a locus control region.

9. A vector according to claim 8, wherein the locus control region is the CD2 LCR.

10. A vector according to anyone of the aforegoing claims additionally comprising further regulatory elements such as boundary elements, tissue specific promoters or enhancers and the like.

11. A vector based on a vector according to anyone of the aforegoing claims wherein at least one gene of interest has been inserted in the site present therefor.

12. A vector according to claim 11, wherein a gene of interest is a selection marker gene.

13. A vector according to claim 12, wherein the selection marker gene is the neomycine gene, the DHFR gene, the MDR or hygromycine gene,.

14. A vector according to anyone of the claims 11-13 wherein at least two genes of interest are inserted, the genes being present in a di-or multicistronic unit, the cistrons being separated by a short non-coding linker having a length which is a number of bases dividable by three.

15. A vector according to anyone of claims 11-14 comprising a suicide gene.

16. A vector according to claim 15 comprising a Herpes Simplex virus (HSV) thymidine kinase gene or a cytosine deaminase gene.

17. A vector according to anyone of claims 11-14 comprising a human multidrug resistance gene or a glucocerebrosidase gene, or HIV sequences leading to inhibition of replication.

18. A kit of parts comprising a vector according to anyone of the aforegoing cells and a packaging cell line for such vectors.

19. A kit according to claim 18 wherein the packaging cell line expresses the retroviral proteins necessary for producing a virus-like particle.

20. A virus-like particle comprising a vector according to anyone of the aforegoing claims.

21. A method of providing cells with genetic material of interest comprising contacting such cells with a virus-like particle according to claim 20.

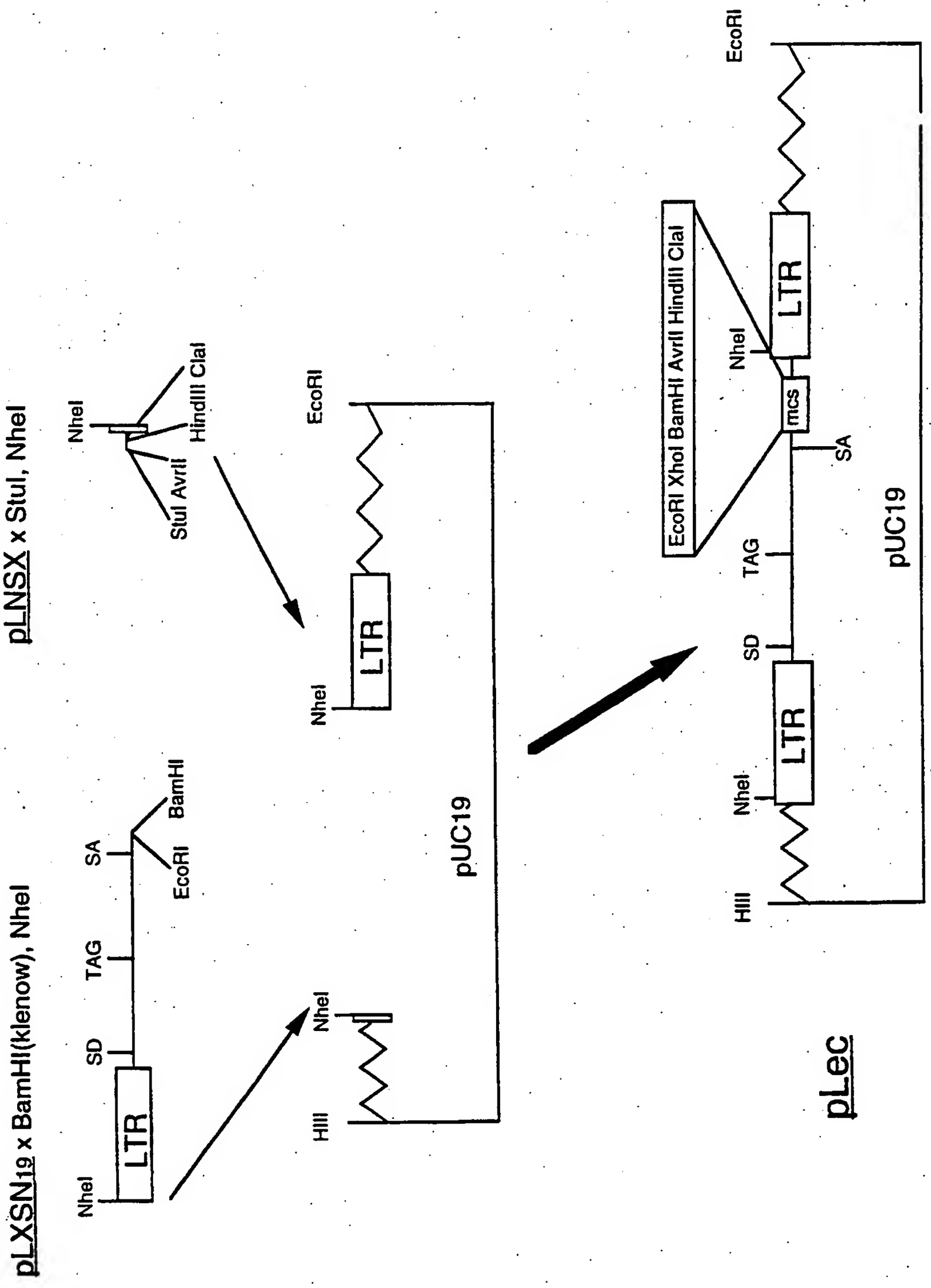


FIG. 1 : construction of pLec

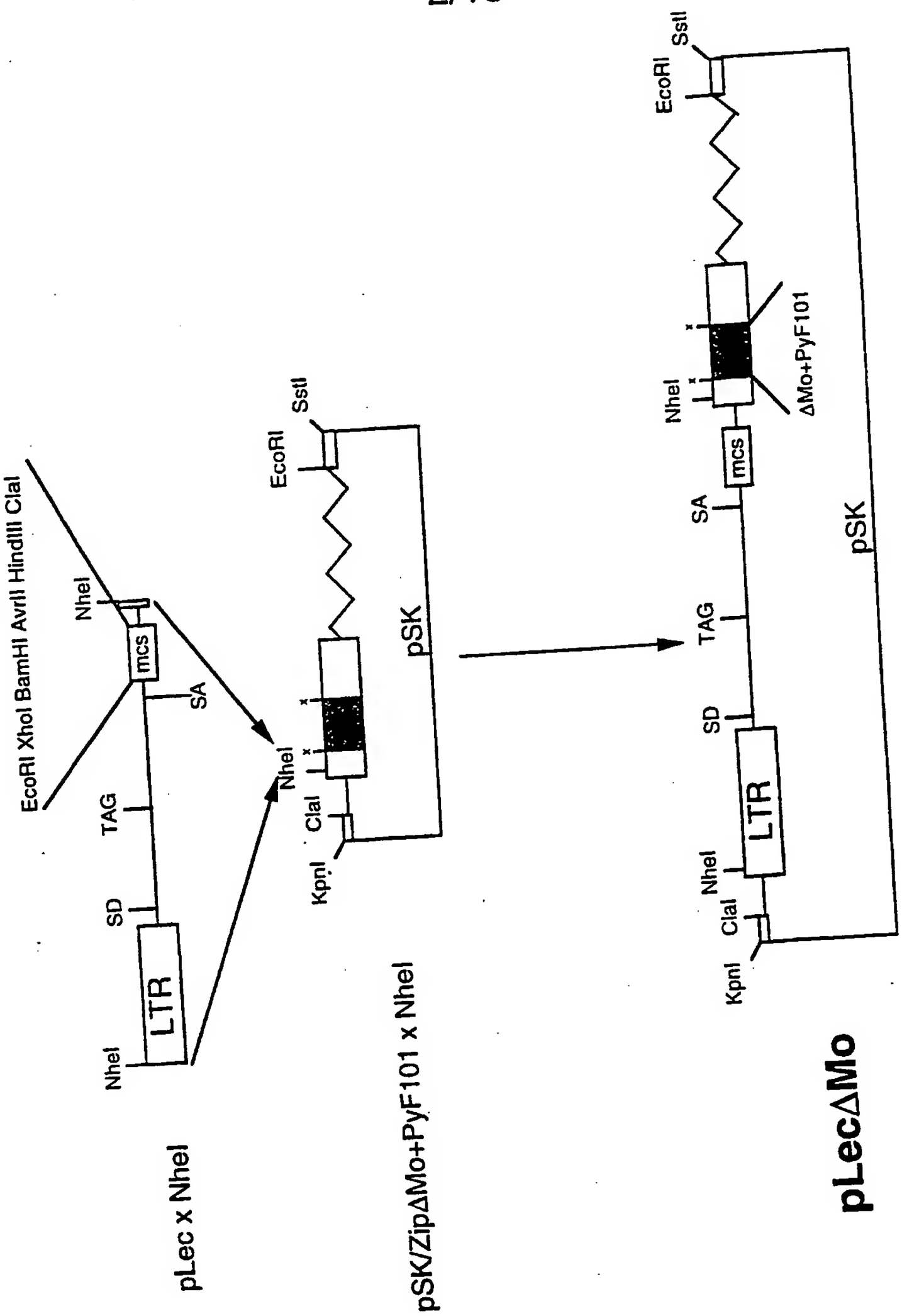


FIG. 2: construction of pLecΔMo

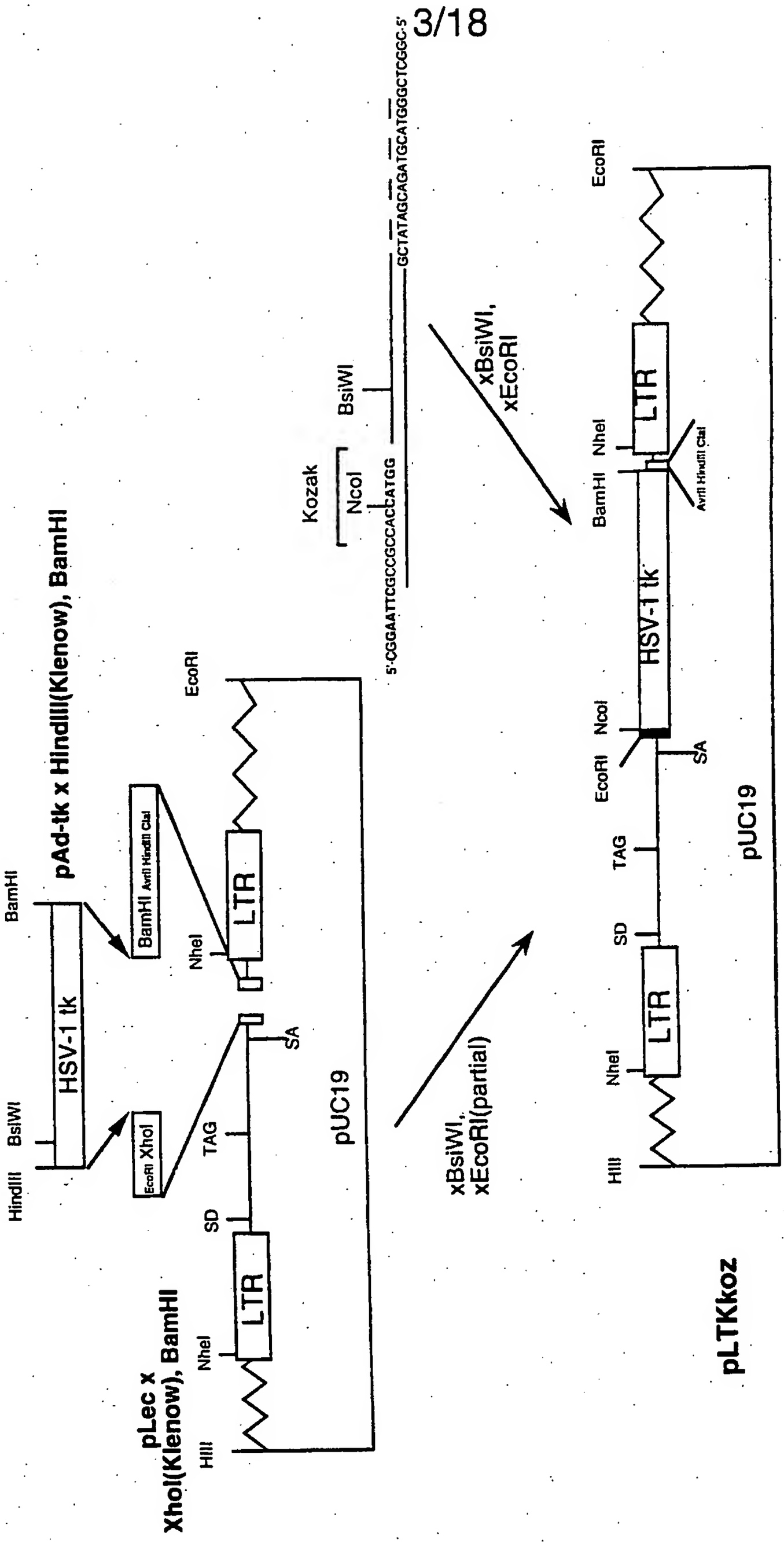


FIG. 3 : construction of pLTKkoz

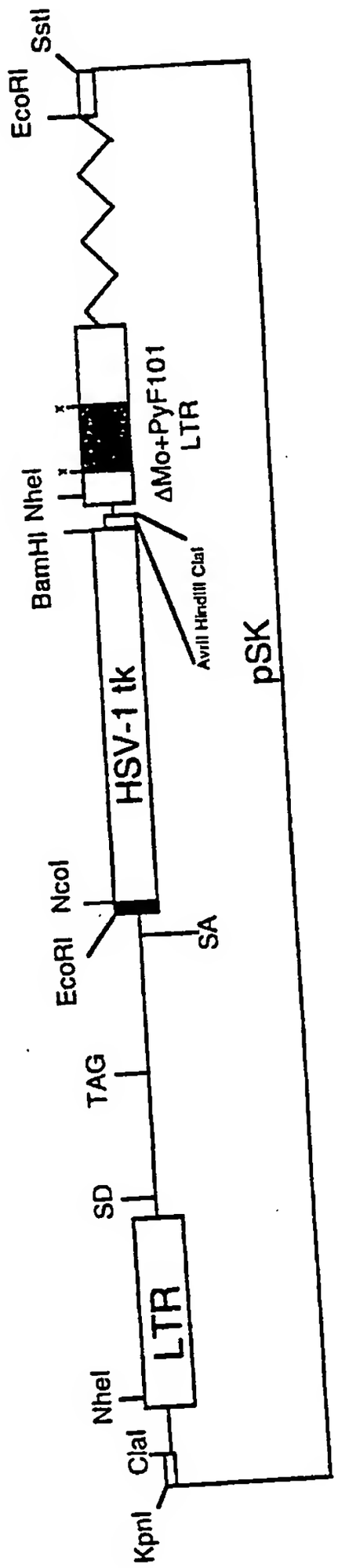


FIG. 4a: map of pIGTK

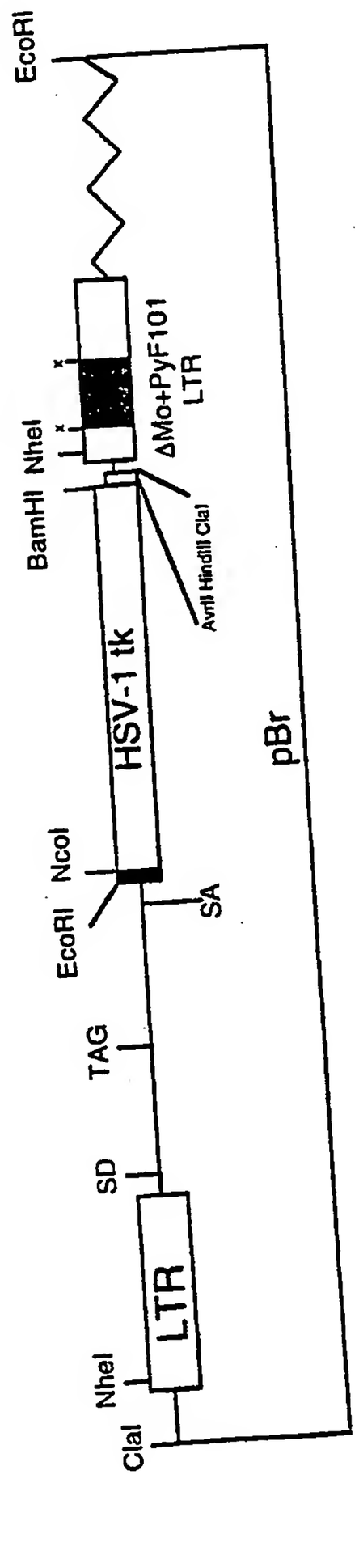


FIG. 4b: map of pLTKΔMo

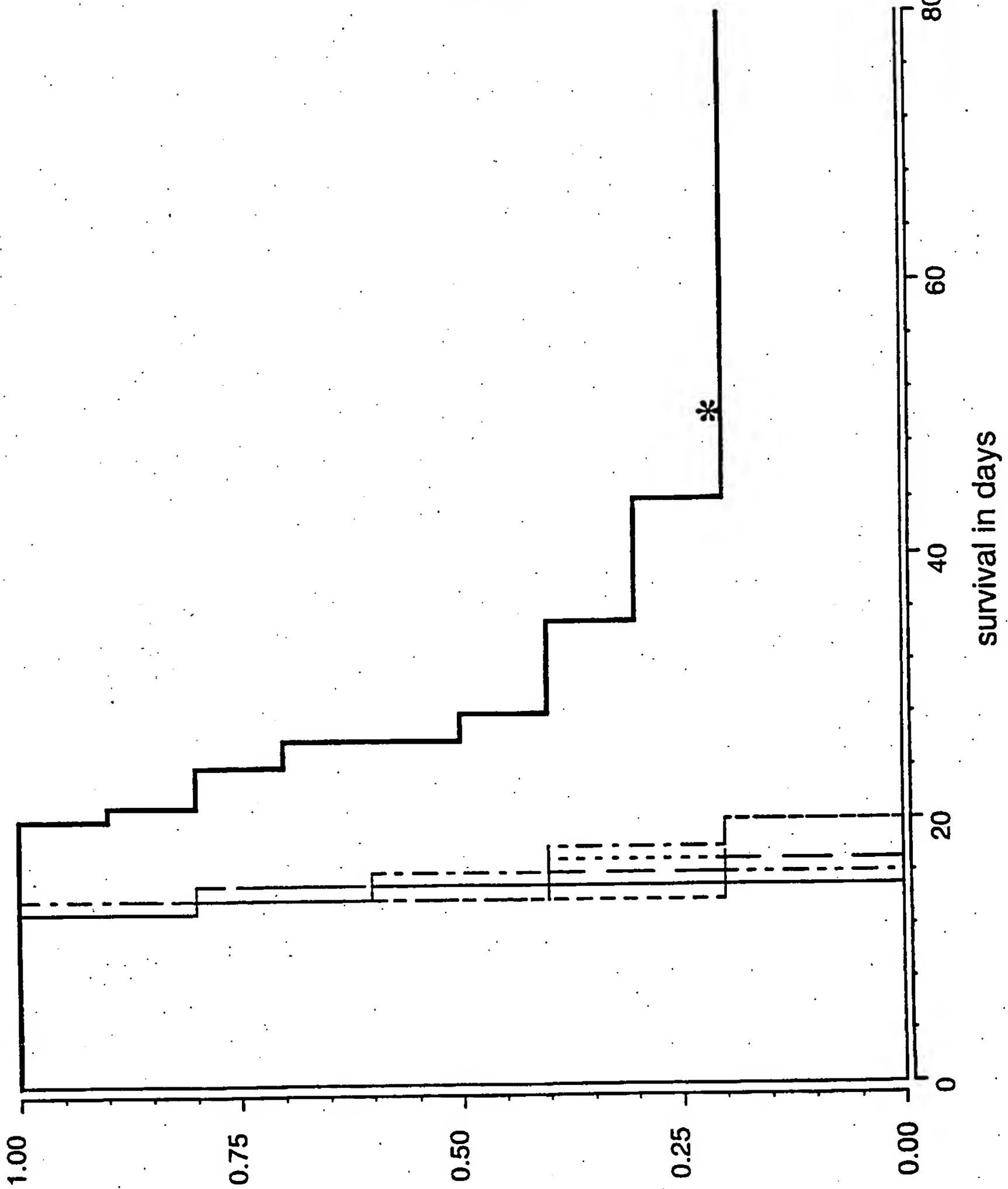

survival in days
0
20
40
60
80
surviving fraction
0.00
0.25
0.50
0.75
1.00

FIG. 5.

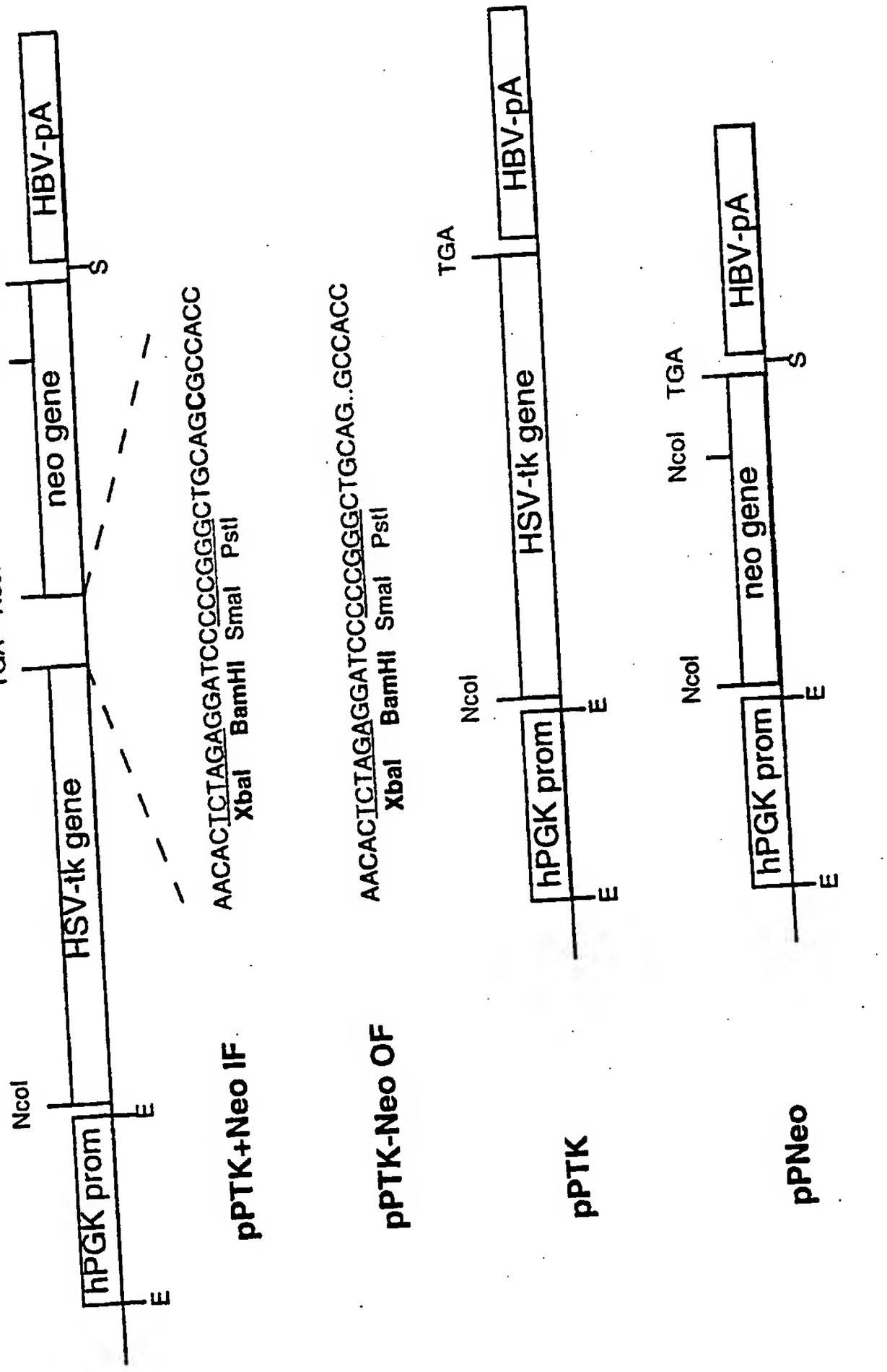

pPTK+Neo IF
pPTK-Neo OF
pPTK
pPNeo
HSV-tk gene
neo gene
hPGK prom
HBV-pA
TGA
NcoI
E
S
AACACICTAGAGGATCCCCCGGGCTGCAGCGCCACC
XbaI BamHI SmaI PstI
AACACICTAGAGGATCCCCCGGGCTGCAG..GCCACC
XbaI BamHI SmaI PstI

FIG. 6

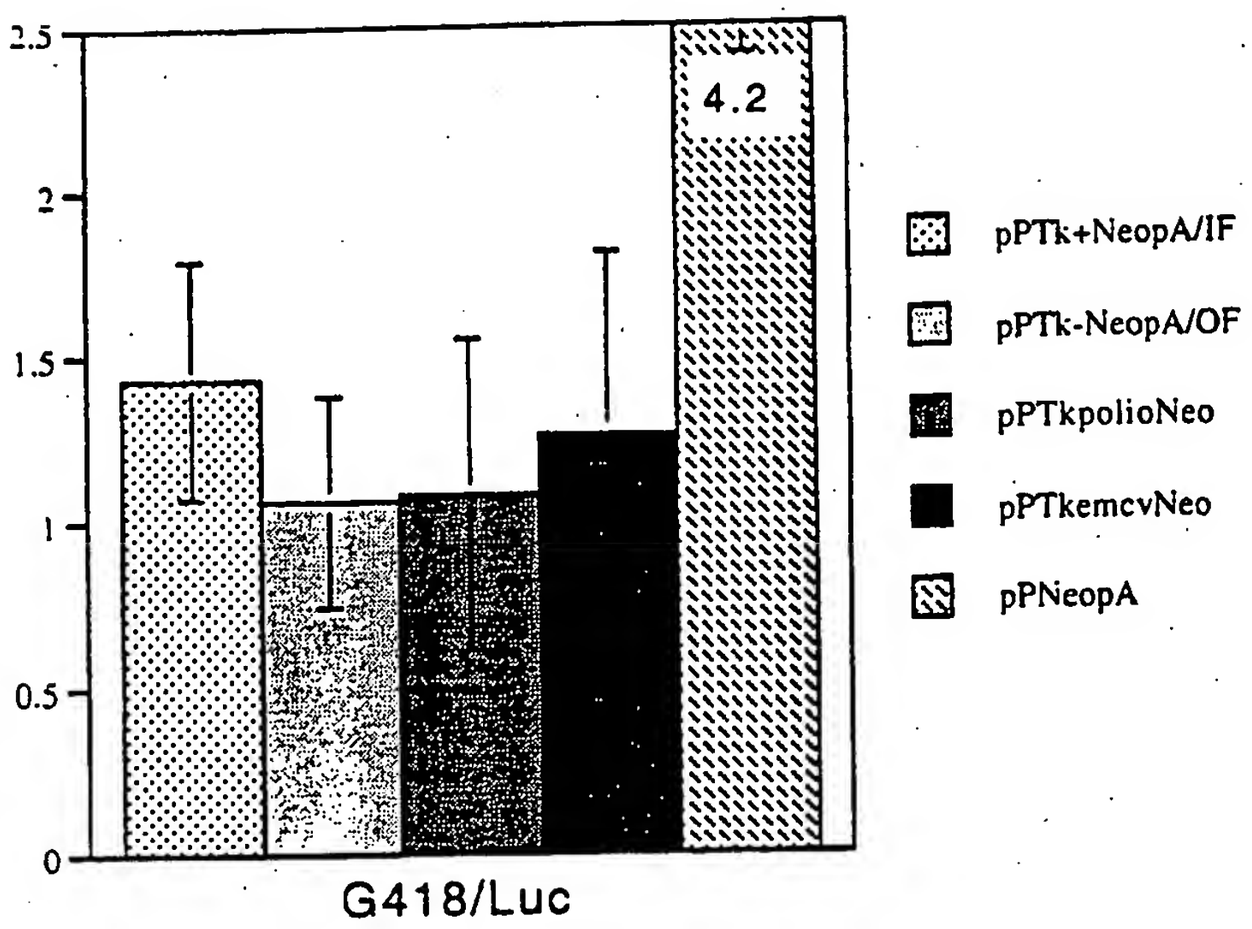

2.5
2
1.5
1
0.5
0
4.2
pPTk+NeopA/IF
pPTk-NeopA/OF
pPTkpolioNeo
pPTkemcvNeo
pPNeopA
G418/Luc

FIG. 6A

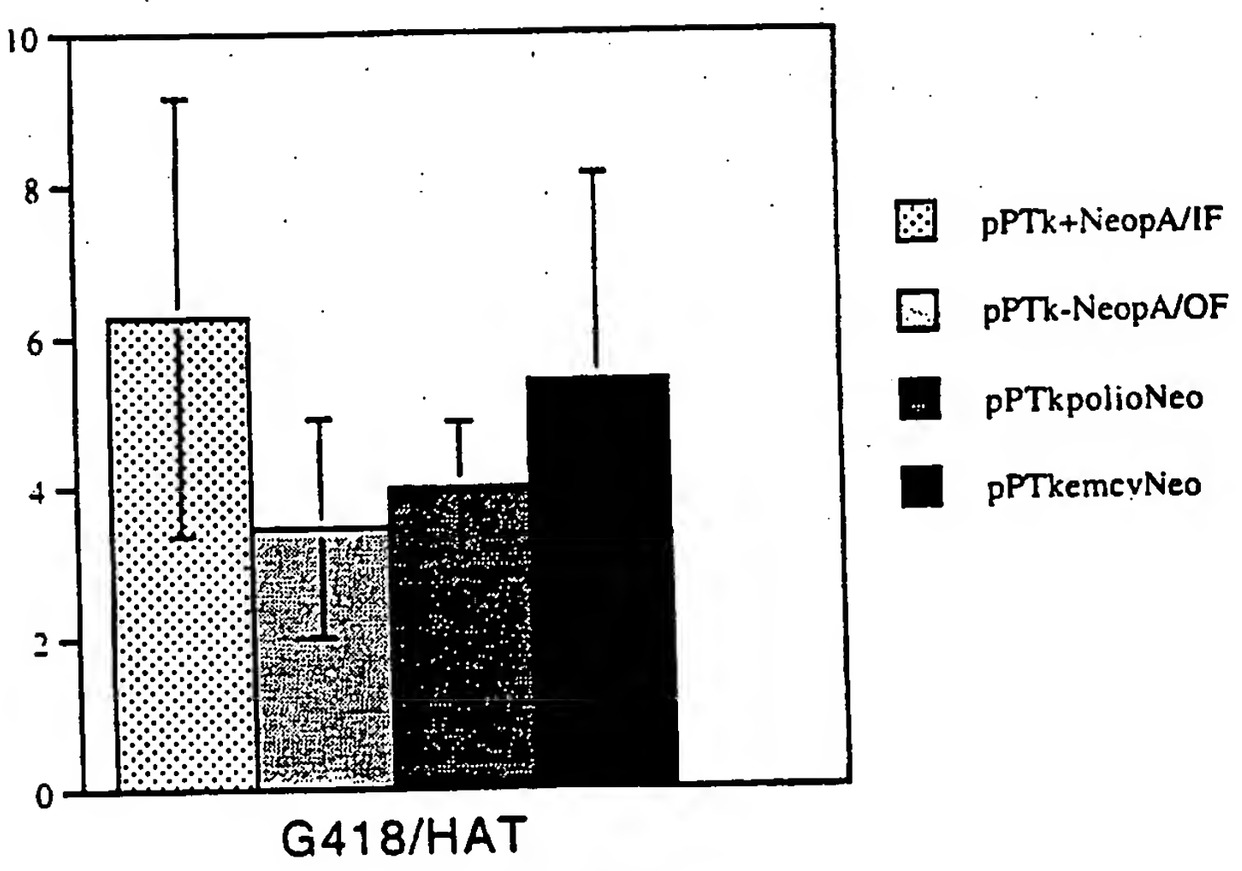

10
8
6
4
2
0
pPTk+NeopA/IF
pPTk-NeopA/OF
pPTkpolioNeo
pPTkemcvNeo
G418/HAT

FIG. 6B

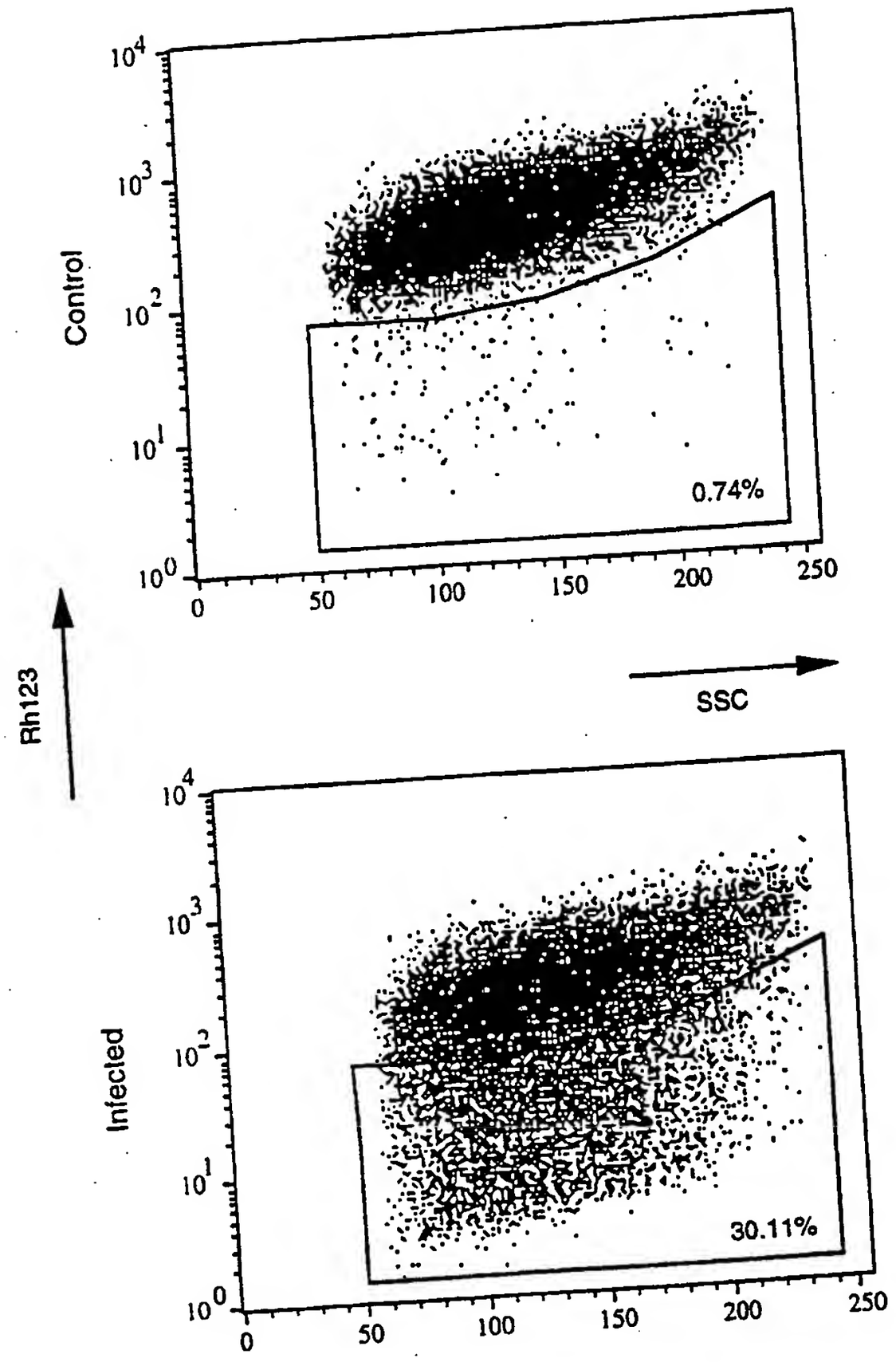


FIG. 7: Rh123 exclusion assay following bulk-infection of A2780 cells with IGmdr1

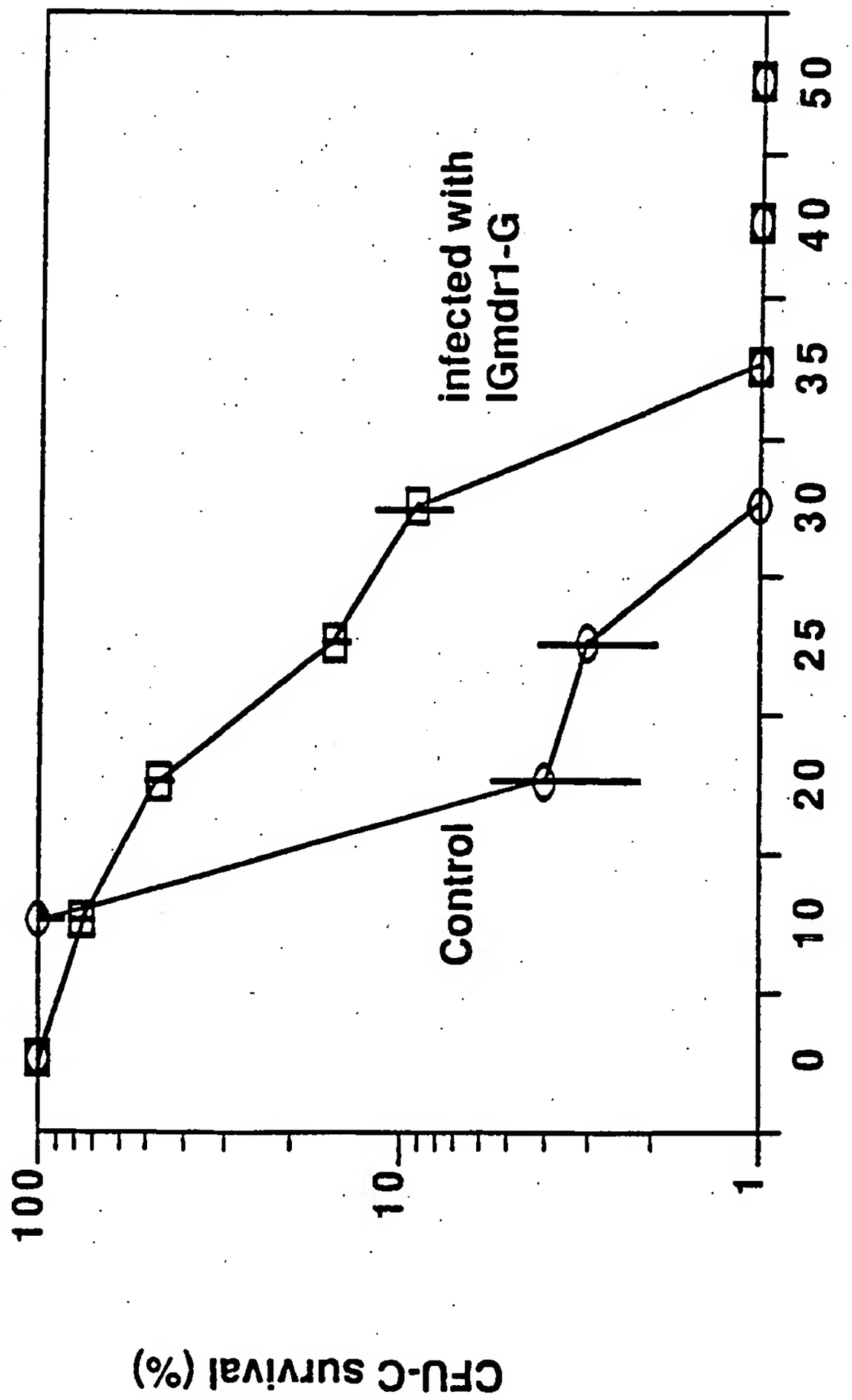


FIG. 8 Reversal of chemotherapy sensitivity in human hemopoietic cells after supernatant transduction with IGmdr-G vector.

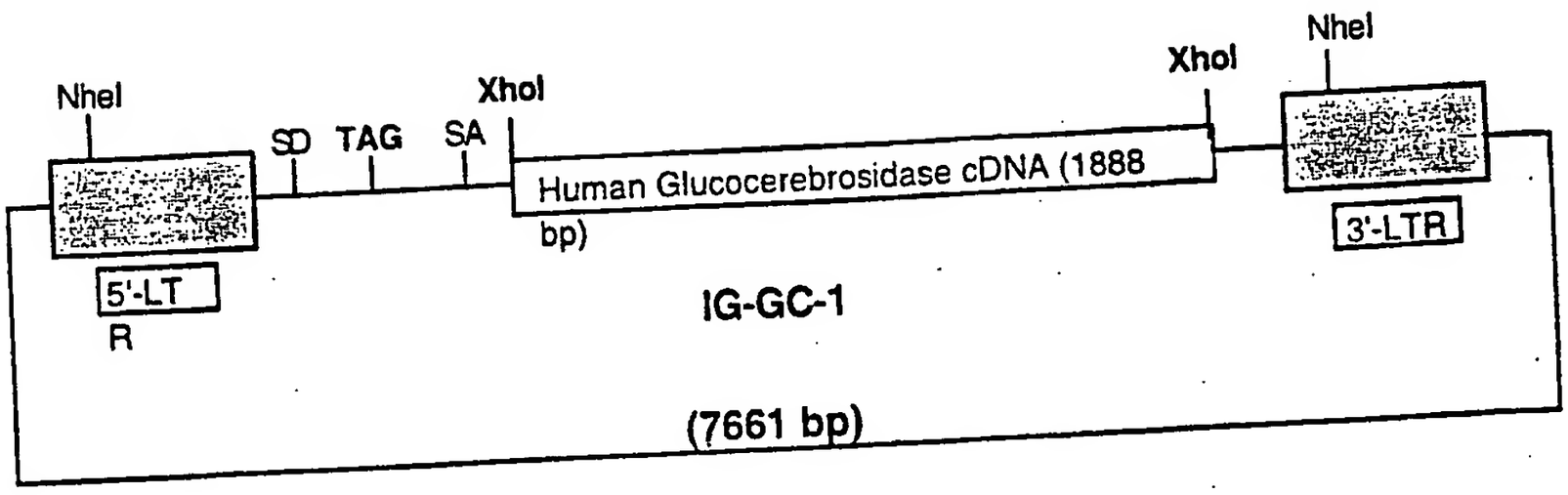


FIG. 9 Physical map of retroviral construct IG-GC-1.

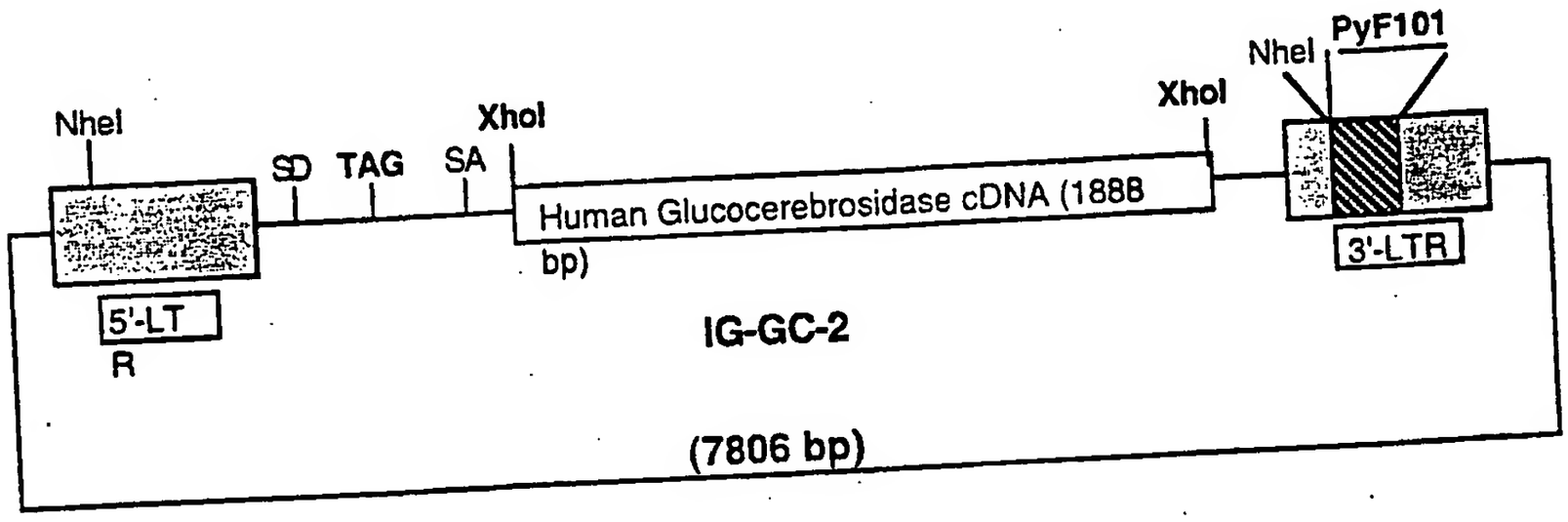


FIG. 10 Physical map of retroviral construct IG-GC-2.

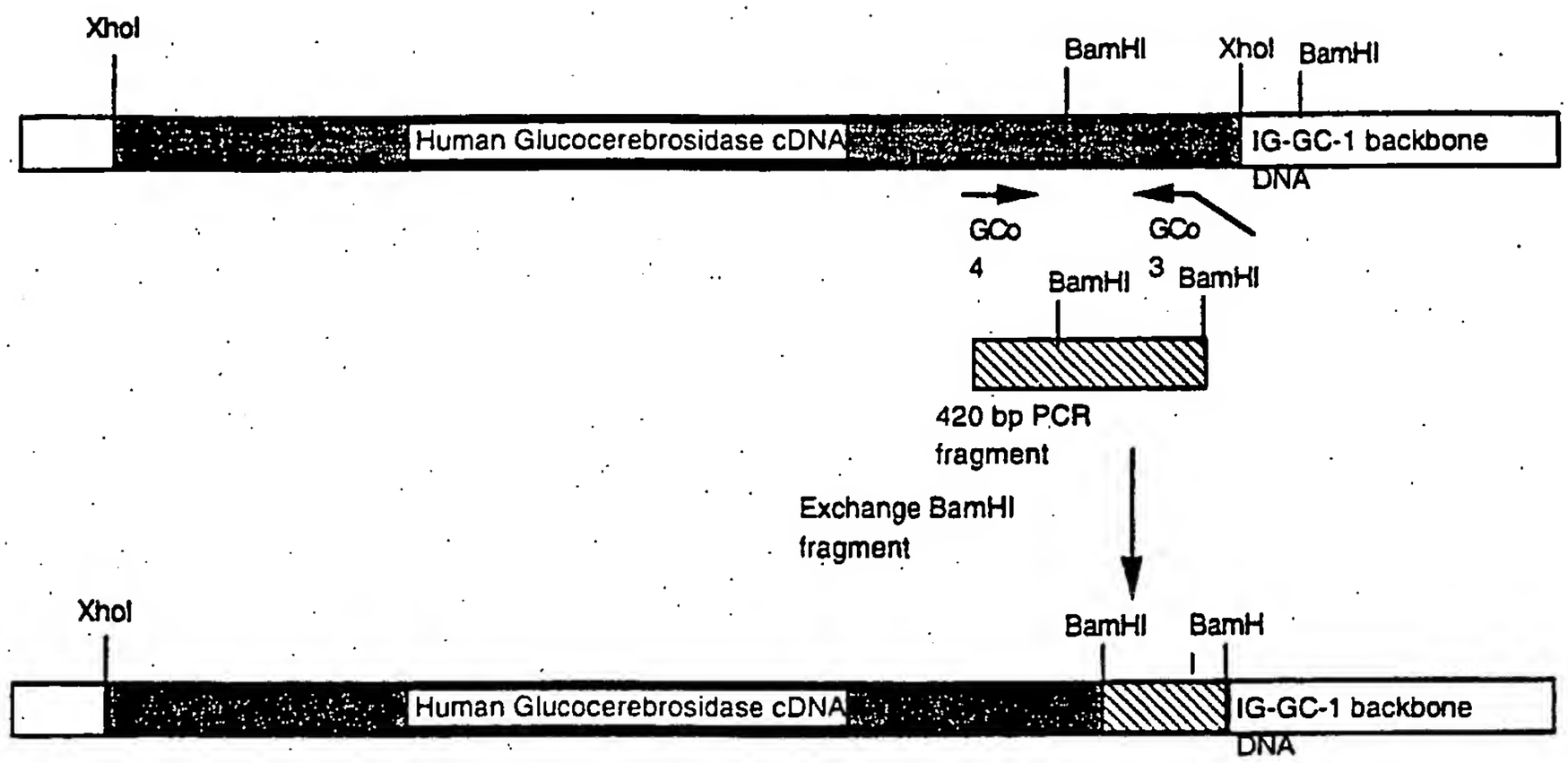


FIG. 11 PCR strategy to obtain IG-GC-3 (see text for details)

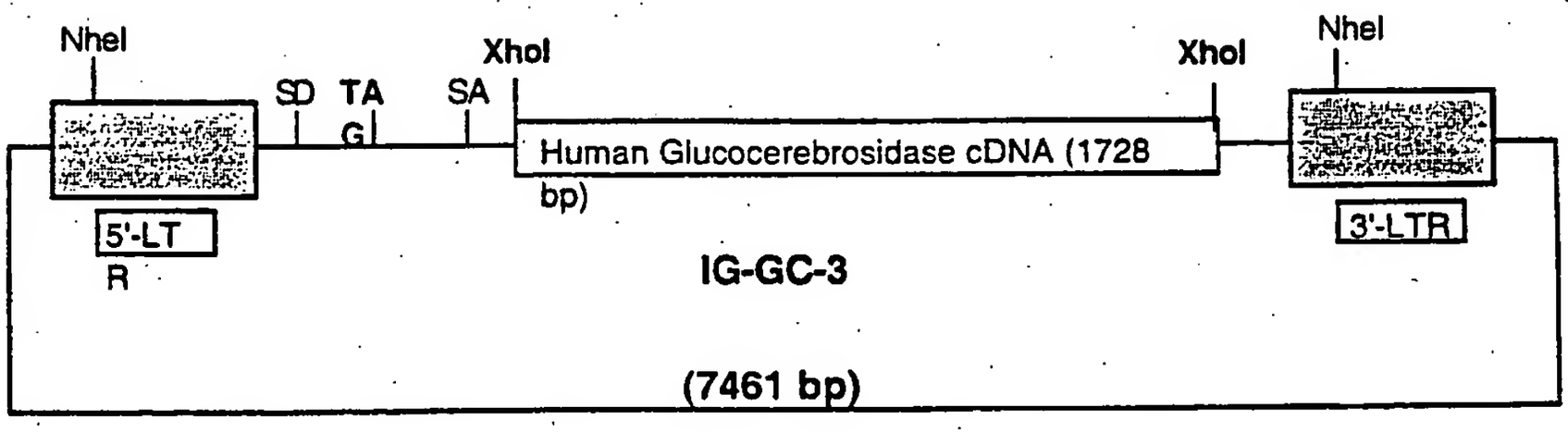


FIG. 12 Physical map of retroviral construct IG-GC-3.

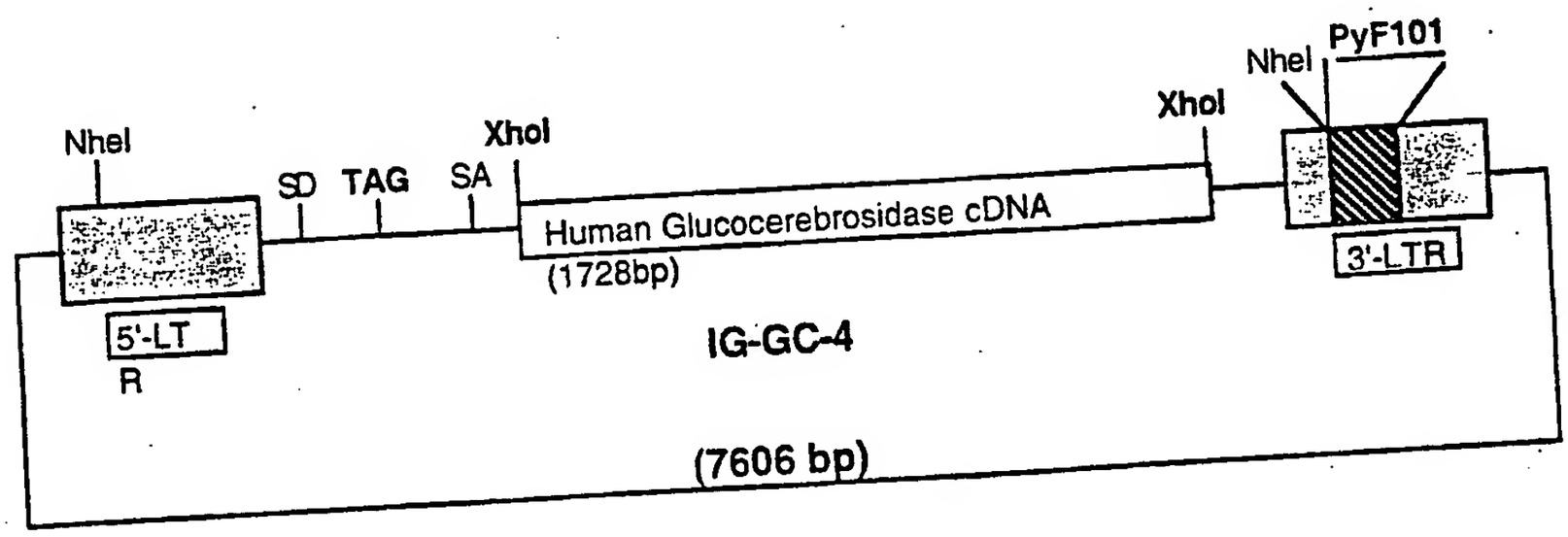


FIG. 13 Physical map of retroviral construct IG-GC-4.

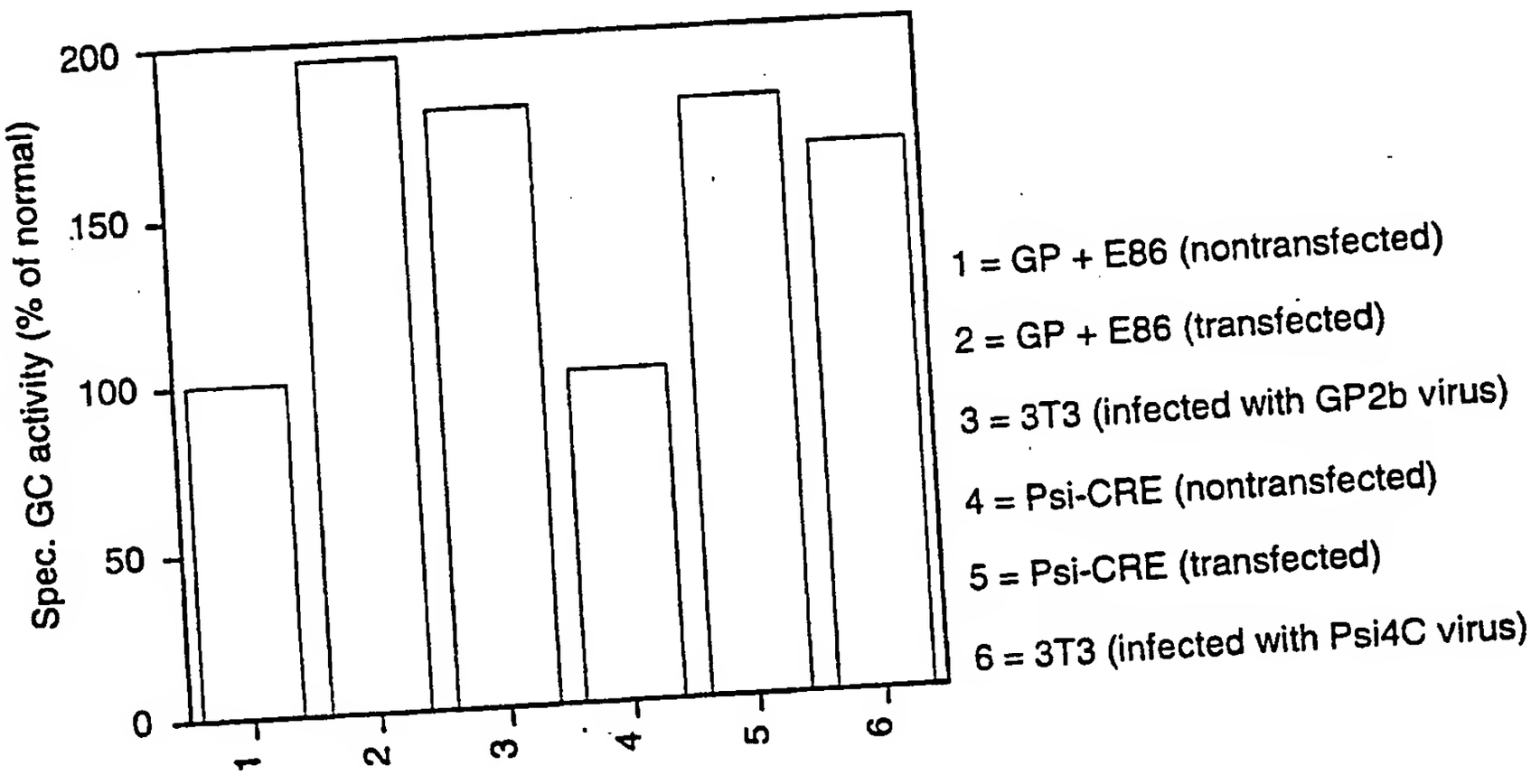


FIG. 14A Increase in GC enzymatic activity after transfection of retroviral vector IG-GC-2 (column 2 and 5) and after infection of IG-GC-2 recombinant ecotropic virus (column 3 and 6).

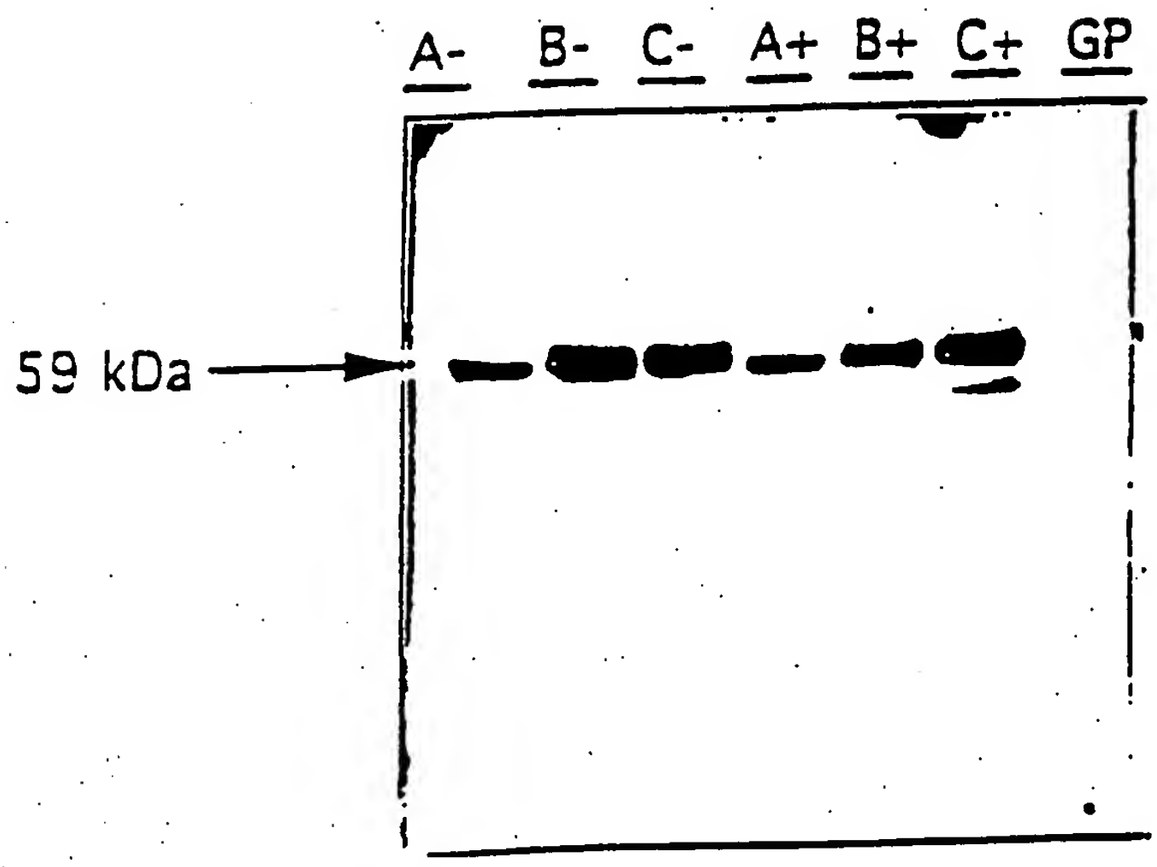


FIG. 14B Western blot (80 119 total protein/lane) with human GC specific monoclonal antibody 8E4. A, B, C: cell lysates prepared from GP + E86 cells after transfection with retroviral vector IG-GC-2. -/+ with or without protease inhibitors. GP: cell lysate prepared from non-infected GP + E86 cells.

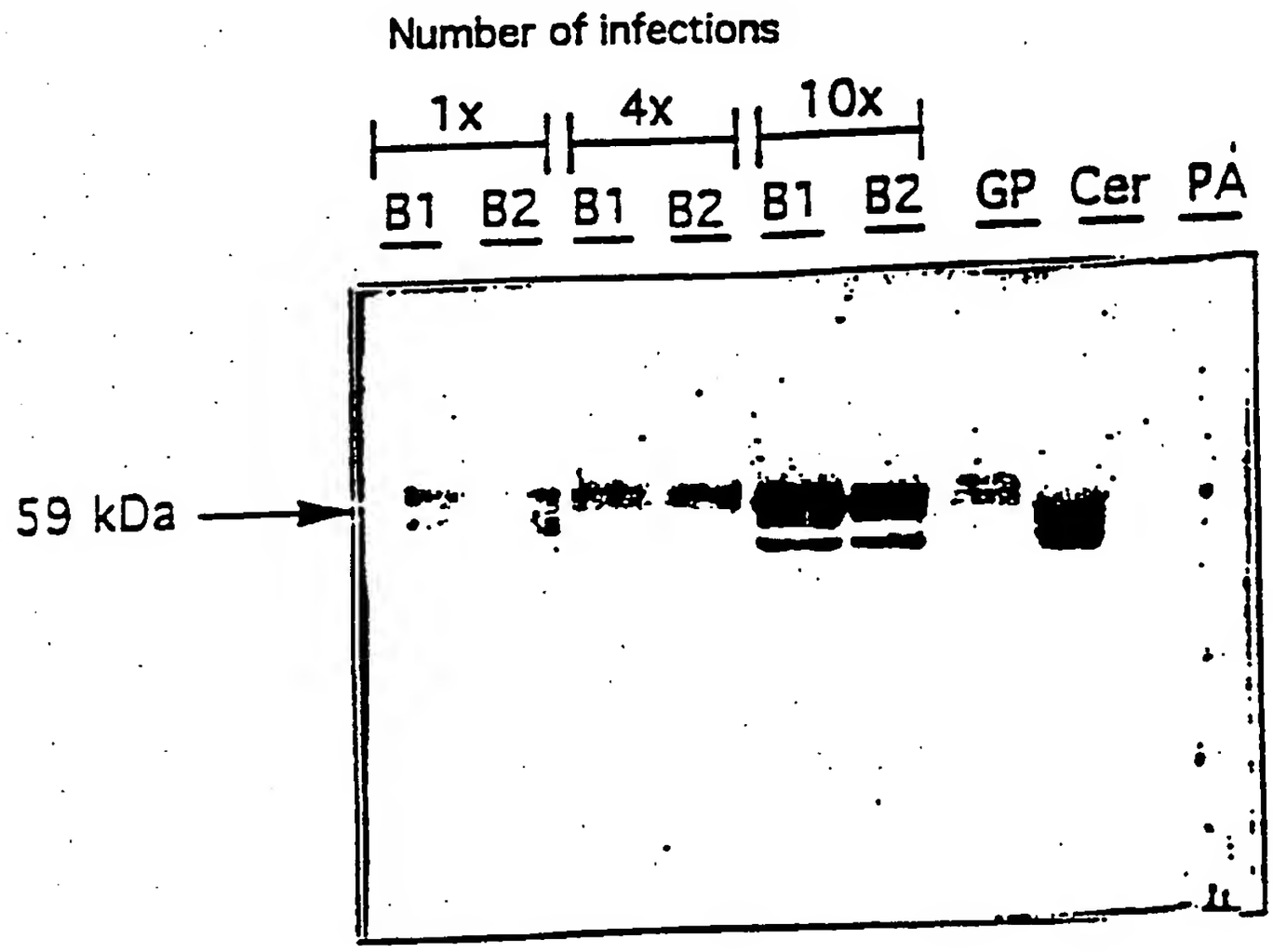


Fig. 15 Western blot (80 µg total protein/lane) with human GC specific monoclonal antibody 8E4. **B1/B2**: cell lysate prepared from PA317 cells after repeated infection (1, 4, 10 times) with ecotropic GP2b virus (duplicates). **GP**: cell lysate prepared from GP + E86 cells after transfection with retroviral construct IG-GC-2. **PA**: cell lysate prepared from non-infected PA3 17 cells. **Cer**: 16.5 mg (0.7 Units) Recombinant GC (Genzyme Corp.) as positive control.

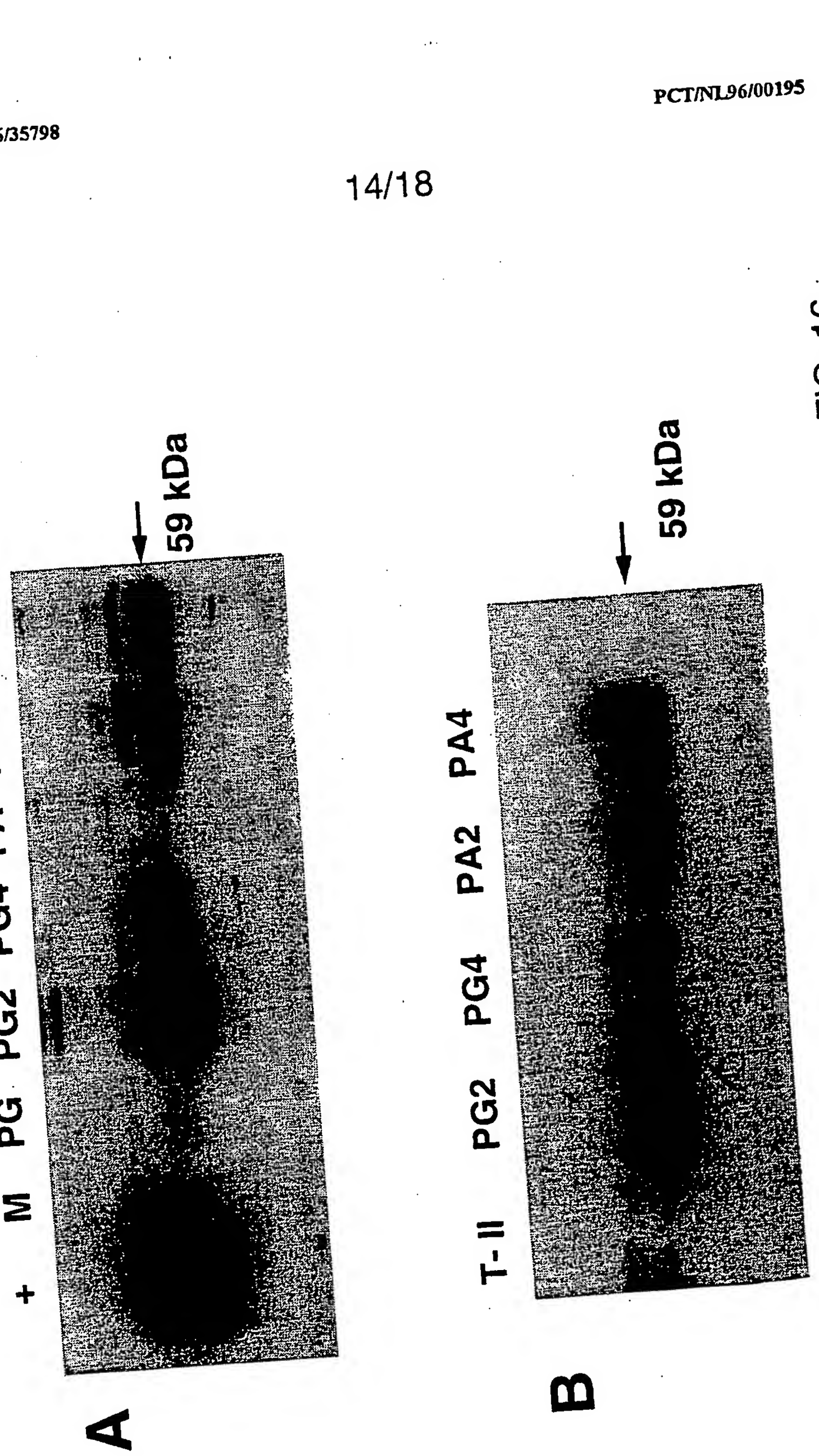


FIG. 16

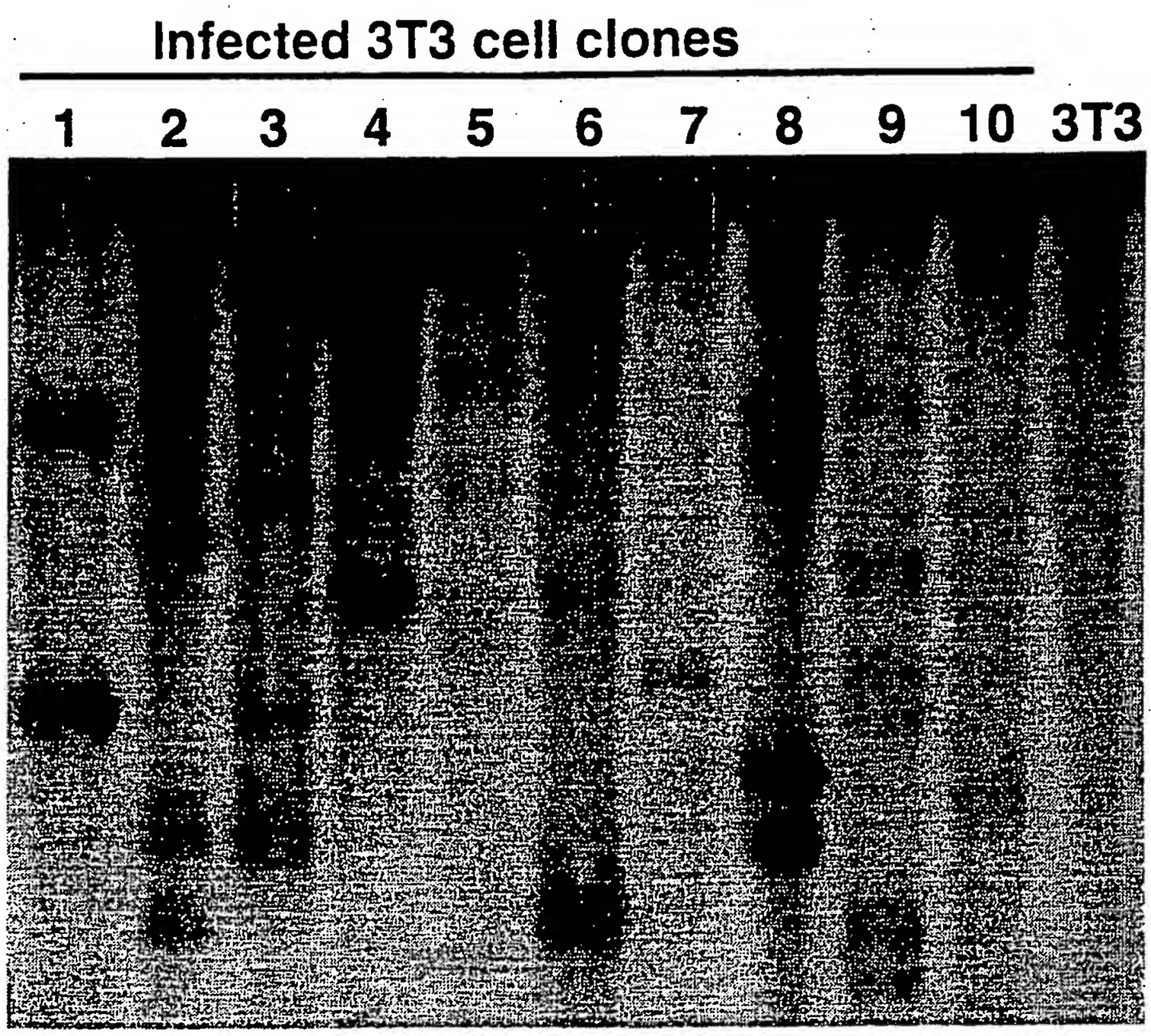
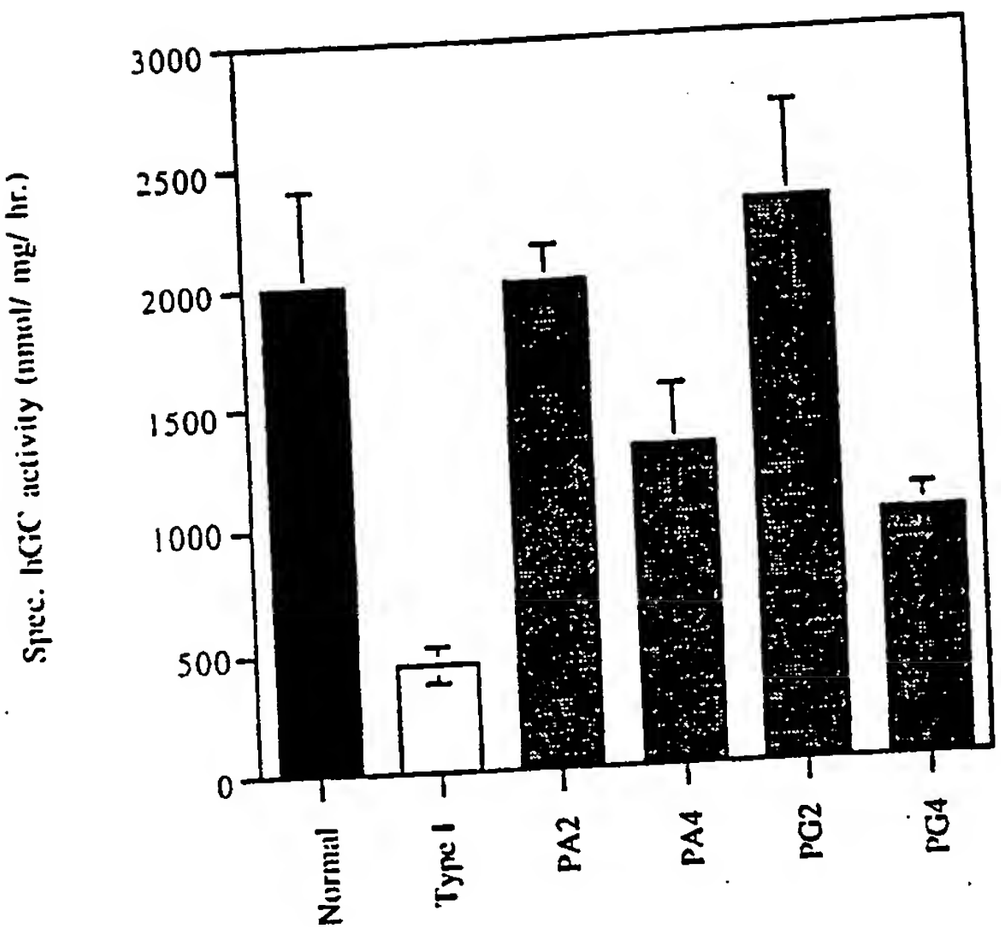


FIG. 17

A


Spec. hGC activity (nmol/ mg/ hr.)
3000
2500
2000
1500
1000
500
0
Normal
Type I
PA2
PA4
PG2
PG4

B

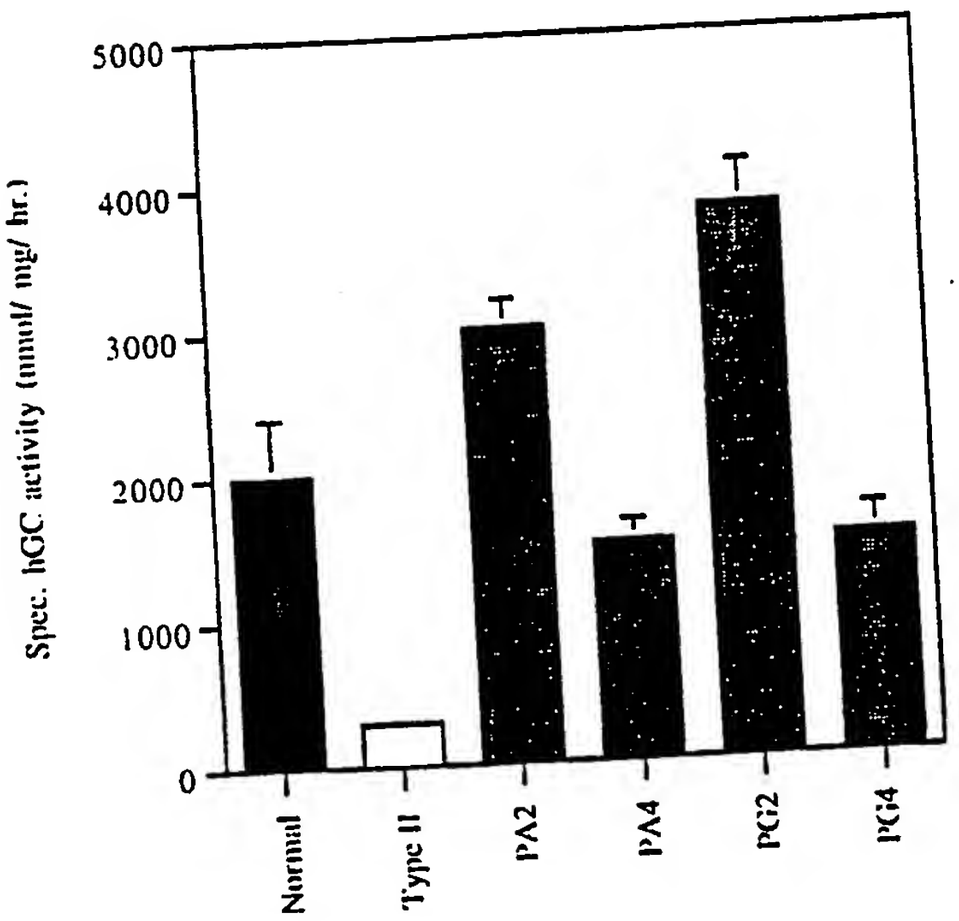

Spec. hGC activity (nmol/ mg/ hr.)
5000
4000
3000
2000
1000
0
Normal
Type II
PA2
PA4
PG2
PG4

FIG. 18

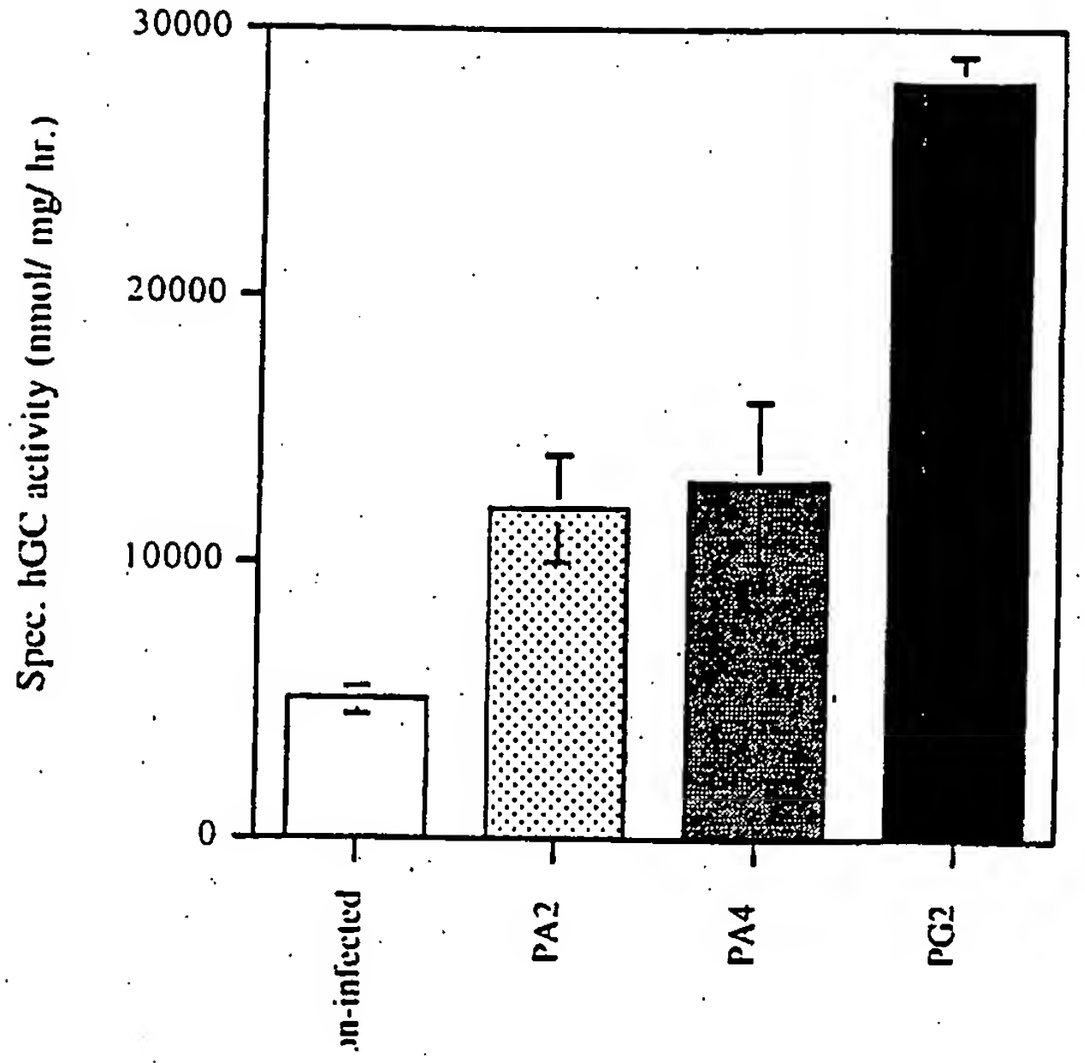

30000
20000
10000
0
Spec. hGC activity (nmol/ mg/ hr.)
PA2
PA4
PG2

FIG. 19

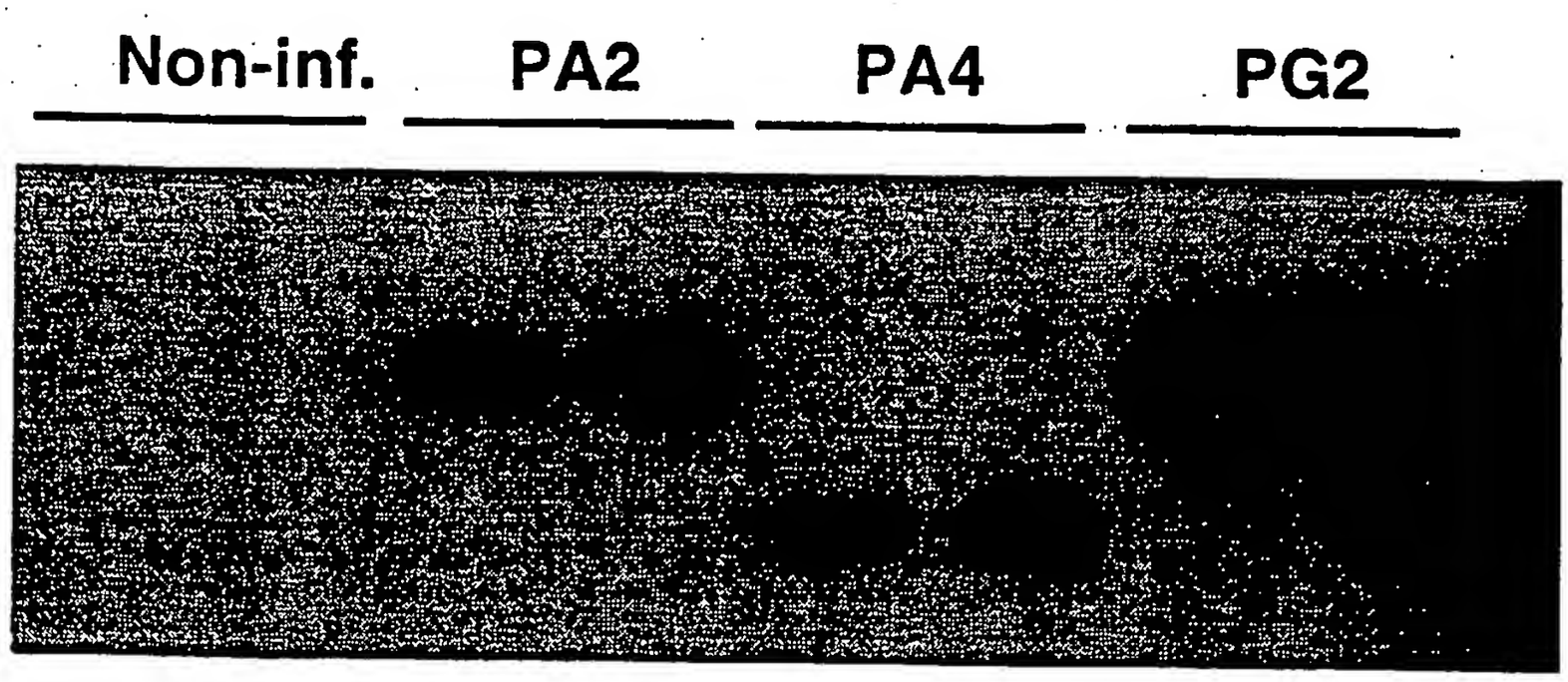

Non-inf.
PA2
PA4
PG2

FIG. 20

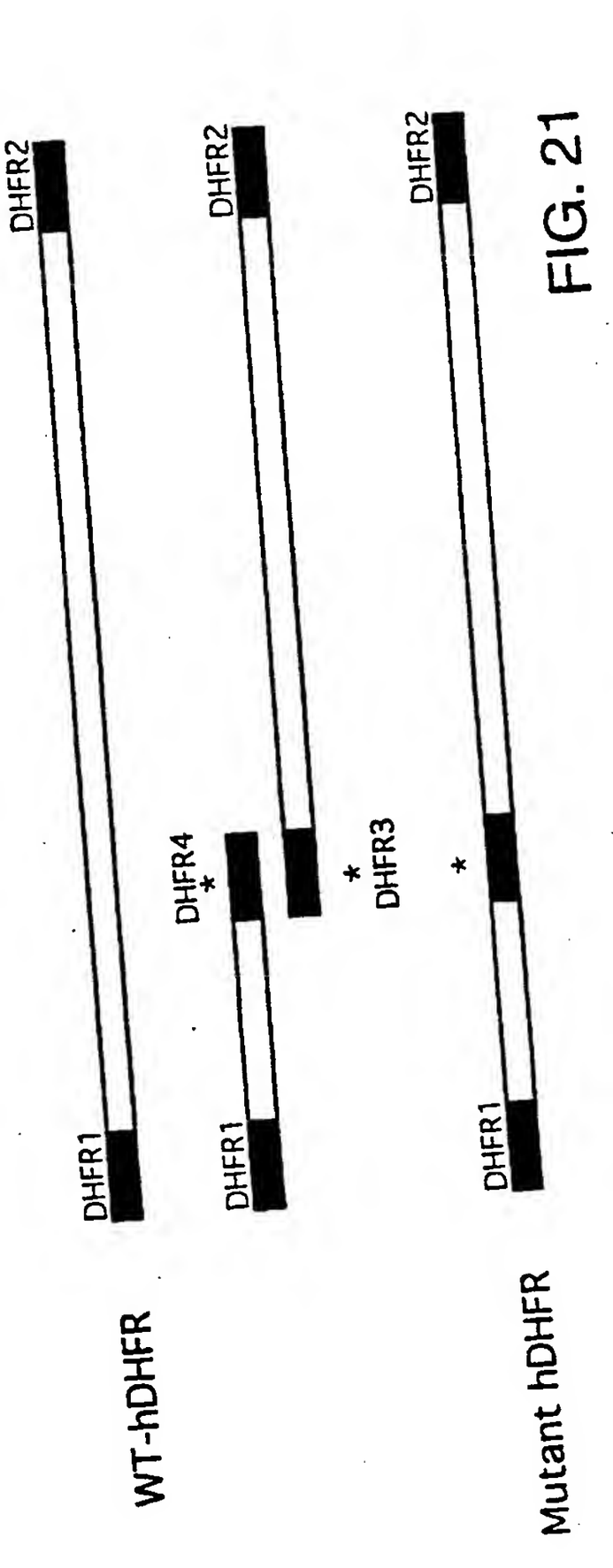
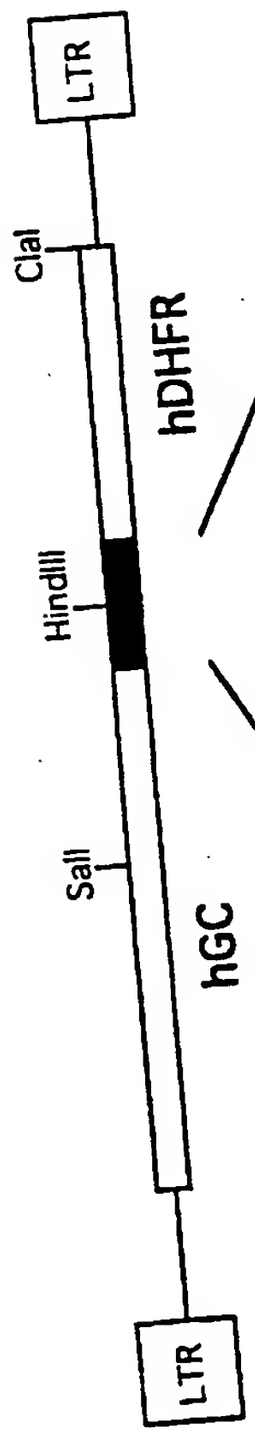

DHFR2
DHFR1
DHFR4
DHFR2
DHFR1
DHFR3
DHFR2
DHFR1
WT-hDHFR
Mutant hDHFR

FIG. 21


LTR
ClaI
HindIII
hDHFR
SalI
hGC
LTR


Kozack
SmaI
HindIII
XbaI
BamHI
TGAGGATCCTCTAGAAGCTTCCCGGGCTGCAGGCCACCATG
STOP-GC
START hDHFR

FIG. 22

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 96/00195

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N7/04 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENE THERAPY, vol. 1, no. 2, March 1994, pages 136-138, XP000601445 HAWLEY, R.G. ET AL.: "Versatile retroviral vectors for potentiel use in gene therapy"	1-4,6, 10-13
Y	see the whole document	1-6, 10-21

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1996	03.09.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	**Authorized officer** Chambonnet, F

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 96/00195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENE, vol. 84, no. 2, 1989, AMSTERDAM NL, pages 419-427, XP002011365 VALERIO, D. ET AL.: "Retrovirus mediated gene transfer into embryonal carcinoma and hemopoietic stem cells: expression from a hybrid long terminal repeat" cited in the application see page 420, column 1, paragraph 2 - column 2, paragraph 2; figure 1 see page 425, column 1, paragraph 3 - page 426, paragraph END ---	1-6, 11-13
Y		1-6, 10-21
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 187, no. 1, 31 August 1992, ORLANDO, FL US, pages 187-194, XP000578334 WILKE, M. ET AL.: "Amphotropic retroviruses with a hybrid long terminal repeat as a tool for gene therapy of cystic fibrosis" see the whole document ---	1-6,11, 12,18-21
Y	WO,A,93 07281 (TNO) 15 April 1993 cited in the application see the whole document ---	1-6, 10-21
X	US,A,4 959 313 (TAKETO MAKOTO) 25 September 1990 see the whole document ---	1-4, 11-13, 20,21
X	WO,A,94 13824 (UNIV PARIS CURIE ;KLATZMANN DAVID (FR); CARUSO MANUEL (FR)) 23 June 1994 see the whole document ---	1-4,6, 10-12, 15,16, 18-21
Y	NATURE, vol. 308, 29 March 1984, LONDON GB, pages 470-472, XP002011366 LINNEY, E. ET AL.: "Non-function of a Moloney murine leukemia virus regulatory sequence in F9 embryonal carcinoma cells" cited in the application see the whole document --- -/--	1-6, 13-21

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCI/NL 96/00195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOTECHNIQUES, vol. 7, no. 9, October 1989, pages 980-990, XP002011367 MILLER, A.D. & ROSMAN, G.J.: "Improved retroviral vectors for gene transfer and expression" cited in the application see the whole document	1-6
Y	WO,A,94 29437 (UNIVERSITY OF MEDICINE & DENTISTRY OF NEW JERSEY) 22 December 1994 see the whole document	2
A	WO,A,94 21806 (MEDICAL RESEARCH COUNCIL & THEREXSYS) 29 September 1994 see the whole document	8,9
A	WO,A,94 29470 (MASSACHUSETTS INST TECHNOLOGY) 22 December 1994 see the whole document	8,9
E	WO,A,96 19245 (LOS ANGELES CHILDRENS HOSPITAL ;KOHN DONALD B (US); CHALLITA PIA M) 27 June 1996 see the whole document	1

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INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/NL96/00195

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : For claim 21 as far as directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]mal Application No
PC[illegible]/NL 96/00195

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9307281	15-04-93	NL-A-	9101680	03-05-93
		AU-B-	2768992	03-05-93
		CA-A-	2120370	15-04-93
		EP-A-	0606376	20-07-94
		JP-T-	7501690	23-02-95
US-A-4959313	25-09-90	NONE		
WO-A-9413824	23-06-94	FR-A-	2699191	17-06-94
		CA-A-	2150536	23-06-94
		EP-A-	0674716	04-10-95
		JP-T-	8506722	23-07-96
WO-A-9429437	22-12-94	NONE		
WO-A-9421806	29-09-94	AU-B-	6261494	11-10-94
		CA-A-	2158252	29-09-94
		EP-A-	0689602	03-01-96
WO-A-9429470	22-12-94	CA-A-	2164953	22-12-94
		EP-A-	0706575	17-04-96
WO-A-9619245	27-06-96	NONE		